COMPOSITIONS AND METHODS FOR WT1 SPECIFIC IMMUNOTHERAPY

TECHNICAL FIELD

The present invention relates generally to the immunotherapy of malignant diseases such as leukemia and cancers. The invention is more specifically related to compositions for generating or enhancing an immune response to WT1, and to the use of such compositions for preventing and/or treating malignant diseases.

BACKGROUND OF THE INVENTION

Cancer and leukemia are significant health problems in the United States and throughout the world. Although advances have been made in detection and treatment of such diseases, no vaccine or other universally successful method for prevention or treatment of cancer and leukemia is currently available. Management of the diseases currently relies on a combination of early diagnosis and aggressive treatment, which may include one or more of a variety of treatments such as surgery, radiotherapy, chemotherapy and hormone therapy. The course of treatment for a particular cancer is often selected based on a variety of prognostic parameters, including an analysis of specific tumor markers. However, the use of established markers often leads to a result that is difficult to interpret, and the high mortality continues to be observed in many cancer patients.

Immunotherapies have the potential to substantially improve cancer and leukemia treatment and survival. Recent data demonstrate that leukemia can be cured by immunotherapy in the context of bone marrow transplantation (*e.g.,* donor lymphocyte infusions). Such therapies may involve the generation or enhancement of an immune response to a tumor-associated antigen (TAA). However, to date, relatively few TAAs are known and the generation of an immune response against such antigens has, with rare exceptions, not been shown to be therapeutically beneficial.

Accordingly, there is a need in the art for improved methods for leukemia and cancer prevention and therapy. The present invention fulfills these needs and further provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, this invention provides compositions and methods for the diagnosis and therapy of diseases such as leukemia and cancer. In one aspect, the present invention provides polypeptides comprising an immunogenic portion of a native WT1, or a variant thereof that differs in one or more substitutions, deletions, additions and/or insertions such that the ability of the variant to react with antigen-specific antisera and/or T-cell lines or clones is not substantially diminished. Within certain embodiments, the polypeptide comprises no more than 16 consecutive amino acid residues of a native WT1 polypeptide. Within other embodiments, the polypeptide comprises an immunogenic portion of amino acid residues 1 - 174 of a native WT1 polypeptide or a variant thereof, wherein the polypeptide comprises no more than 16 consecutive amino acid residues present within amino acids 175 to 345 of the native WT1 polypeptide. The immunogenic portion preferably binds to an MHC class I and/or class II molecule. Within certain embodiments, the polypeptide comprises a sequence selected from the group consisting of (a) sequences recited in any one or more of Tables II - XLVI, (b) variants of the foregoing sequences that differ in one or more substitutions, deletions, additions and/or insertions such that the ability of the variant to react with antigen-specific antisera and/or T-cell lines or clones is not substantially diminished and (c) mimetics of the polypeptides recited above, such that the ability of the mimetic to react with antigen-specific antisera and/or T cell lines or clones is not substantially diminished.

Within other embodiments, the polypeptide comprises a sequence selected from the group consisting of (a) ALLPAVPSL (SEQ ID NO:34), GATLKGVAA (SEQ ID NO:88), CMTWNQMNL (SEQ ID NOs: 49 and 258), SCLESQPTI (SEQ ID NOs: 199 and 296), SCLESQPAI (SEQ ID NO:198), NLYQMTSQL (SEQ ID NOs: 147 and 284), ALLPAVSSL (SEQ ID NOs: 35 and 255), RMFPNAPYL (SEQ ID NOs: 185 and 293), (b) variants of the foregoing sequences that differ in one or more substitutions, deletions, additions and/or insertions such that the ability of the variant to react with antigen-specific antisera and/or T-cell lines or clones is not substantially diminished and (c) mimetics of the polypeptides recited above, such that the ability of the mimetic to react with antigen-specific antisera

and/or T cell lines or clones is not substantially diminished. Mimetics may comprises amino acids in combination with one or more amino acid mimetics or may be entirely nonpeptide mimetics.

Within further aspects, the present invention provides polypeptides comprising a variant of an immunogenic portion of a WT1 protein, wherein the variant differs from the immunogenic portion due to substitutions at between 1 and 3 amino acid positions within the immunogenic portion such that the ability of the variant to react with antigen-specific antisera and/or T-cell lines or clones is enhanced relative to a native WT1 protein.

The present invention further provides WT1 polynucleotides that encode a WT1 polypeptide as described above.

Within other aspects, the present invention provides pharmaceutical compositions and vaccines. Pharmaceutical compositions may comprise a polypeptide or mimetic as described above and/or one or more of (i) a WT1 polynucleotide; (ii) an antibody or antigen-binding fragment thereof that specifically binds to a WT1 polypeptide; (iii) a T cell that specifically reacts with a WT1 polypeptide or (iv) an antigen-presenting cell that expresses a WT1 polypeptide, in combination with a pharmaceutically acceptable carrier or excipient. Vaccines comprise a polypeptide as described above and/or one or more of (i) a WT1 polynucleotide, (ii) an antigen-presenting cell that expresses a WT1 polypeptide or (iii) an anti-idiotypic antibody, and a non-specific immune response enhancer. Within certain embodiments, less than 23 consecutive amino acid residues, preferably less than 17 amino acid residues, of a native WT1 polypeptide are present within a WT1 polypeptide employed within such pharmaceutical compositions and vaccines. The immune response enhancer may be an adjuvant. Preferably, an immune response enhancer enhances a T cell response.

The present invention further provides methods for enhancing or inducing an immune response in a patient, comprising administering to a patient a pharmaceutical composition or vaccine as described above. In certain embodiments, the patient is a human.

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The present invention further provides methods for inhibiting the development of a malignant disease in a patient, comprising administering to a patient a pharmaceutical composition or vaccine as described above. Malignant diseases include, but are not limited to leukemias (*e.g.*, acute myeloid, acute lymphocytic and chronic myeloid) and cancers (*e.g.*, breast, lung, thyroid or gastrointestinal cancer or a melanoma). The patient may, but need not, be afflicted with the malignant disease, and the administration of the pharmaceutical composition or vaccine may inhibit the onset of such a disease, or may inhibit progression and/or metastasis of an existing disease.

The present invention further provides, within other aspects, methods for removing cells expressing WT1 from bone marrow and/or peripheral blood or fractions thereof, comprising contacting bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood with T cells that specifically react with a WT1 polypeptide, wherein the step of contacting is performed under conditions and for a time sufficient to permit the removal of WT1 positive cells to less than 10%, preferably less than 5% and more preferably less than 1%, of the number of myeloid or lymphatic cells in the bone marrow, peripheral blood or fraction. Bone marrow, peripheral blood and fractions may be obtained from a patient afflicted with a disease associated with WT1 expression, or may be obtained from a human or non-human mammal not afflicted with such a disease.

Within related aspects, the present invention provides methods for inhibiting the development of a malignant disease in a patient, comprising administering to a patient bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood prepared as described above. Such bone marrow, peripheral blood or fractions may be autologous, or may be derived from a related or unrelated human or non-human animal (*e.g.*, syngeneic or allogeneic).

In other aspects, the present invention provides methods for stimulating (or priming) and/or expanding T cells, comprising contacting T cells with a WT1 polypeptide under conditions and for a time sufficient to permit the stimulation and/or expansion of T cells. Such T cells may be autologous, allogeneic, syngeneic or unrelated WT1-specific T cells, and may be stimulated *in vitro* or *in vivo*. Expanded T

cells may, within certain embodiments, be present within bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood, and may (but need not) be clonal. Within certain embodiments, T cells may be present in a mammal during stimulation and/or expansion. WT1-specific T cells may be used, for example, within donor lymphocyte infusions.

Within related aspects, methods are provided for inhibiting the development of a malignant disease in a patient, comprising administering to a patient T cells prepared as described above. Such T cells may, within certain embodiments, be autologous, syngeneic or allogeneic.

The present invention further provides, within other aspects, methods for monitoring the effectiveness of an immunization or therapy for a malignant disease associated with WT1 expression in a patient. Such methods are based on monitoring antibody, CD4+ T cell and/or CD8+ T cell responses in the patient. Within certain such aspects, a method may comprise the steps of: (a) incubating a first biological sample with one or more of: (i) a WT1 polypeptide; (ii) a polynucleotide encoding a WT1 polypeptide; or (iii) an antigen presenting cell that expresses a WT1 polypeptide, wherein the first biological sample is obtained from a patient prior to a therapy or immunization, and wherein the incubation is performed under conditions and for a time sufficient to allow immunocomplexes to form; (b) detecting immunocomplexes formed between the WT1 polypeptide and antibodies in the biological sample that specifically bind to the WT1 polypeptide; (c) repeating steps (a) and (b) using a second biological sample obtained from the same patient following therapy or immunization; and (d) comparing the number of immunocomplexes detected in the first and second biological samples, and therefrom monitoring the effectiveness of the therapy or immunization in the patient.

Within certain embodiments of the above methods, the step of detecting comprises (a) incubating the immunocomplexes with a detection reagent that is capable of binding to the immunocomplexes, wherein the detection reagent comprises a reporter group, (b) removing unbound detection reagent, and (c) detecting the presence or absence of the reporter group. The detection reagent may comprise, for example, a

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second antibody, or antigen-binding fragment thereof, capable of binding to the antibodies that specifically bind to the WT1 polypeptide or a molecule such as Protein A. Within other embodiments, a reporter group is bound to the WT1 polypeptide, and the step of detecting comprises removing unbound WT1 polypeptide and subsequently detecting the presence or absence of the reporter group.

Within further aspects, methods for monitoring the effectiveness of an immunization or therapy for a malignant disease associated with WT1 expression in a patient may comprise the steps of: (a) incubating a first biological sample with one or more of: (i) a WT1 polypeptide; (ii) a polynucleotide encoding a WT1 polypeptide; or (iii) an antigen presenting cell that expresses a WT1 polypeptide, wherein the biological sample comprises CD4+ and/or CD8+ T cells and is obtained from a patient prior to a therapy or immunization, and wherein the incubation is performed under conditions and for a time sufficient to allow specific activation, proliferation and/or lysis of T cells; (b) detecting an amount of activation, proliferation and/or lysis of the T cells; (c) repeating steps (a) and (b) using a second biological sample comprising CD4+ and/or CD8+ T cells, wherein the second biological sample is obtained from the same patient following therapy or immunization; and (d) comparing the amount of activation, proliferation and/or lysis of T cells in the first and second biological samples, and therefrom monitoring the effectiveness of the therapy or immunization in the patient.

The present invention further provides methods for inhibiting the development of a malignant disease associated with WT1 expression in a patient, comprising the steps of: (a) incubating $CD4^+$ and/or CD8+ T cells isolated from a patient with one or more of: (i) a WT1 polypeptide; (ii) a polynucleotide encoding a WT1 polypeptide; or (iii) an antigen presenting cell that expresses a WT1 polypeptide, such that the T cells proliferate; and (b) administering to the patient an effective amount of the proliferated T cells, and therefrom inhibiting the development of a malignant disease in the patient. Within certain embodiments, the step of incubating the T cells may be repeated one or more times.

Within other aspects, the present invention provides methods for inhibiting the development of a malignant disease associated with WT1 expression in a

patient, comprising the steps of: (a) incubating $CD4^+$ and/or CD8+ T cells isolated from a patient with one or more of: (i) a WT1 polypeptide; (ii) a polynucleotide encoding a WT1 polypeptide; or (iii) an antigen presenting cell that expresses a WT1 polypeptide, such that the T cells proliferate; (b) cloning one or more cells that proliferated; and (c) administering to the patient an effective amount of the cloned T cells.

Within other aspects, methods are provided for determining the presence or absence of a malignant disease associated with WT1 expression in a patient, comprising the steps of: (a) incubating $CD4^+$ and/or CD8+ T cells isolated from a patient with one or more of: (i) a WT1 polypeptide; (ii) a polynucleotide encoding a WT1 polypeptide; or (iii) an antigen presenting cell that expresses a WT1 polypeptide; and (b) detecting the presence or absence of specific activation of the T cells, therefrom determining the presence or absence of a malignant disease associated with WT1 expression. Within certain embodiments, the step of detecting comprises detecting the presence or absence of proliferation of the T cells.

Within further aspects, the present invention provides methods for determining the presence or absence of a malignant disease associated with WT1 expression in a patient, comprising the steps of: (a) incubating a biological sample obtained from a patient with one or more of: (i) a WT1 polypeptide; (ii) a polynucleotide encoding a WT1 polypeptide; or (iii) an antigen presenting cell that expresses a WT1 polypeptide, wherein the incubation is performed under conditions and for a time sufficient to allow immunocomplexes to form; and (b) detecting immunocomplexes formed between the WT1 polypeptide and antibodies in the biological sample that specifically bind to the WT1 polypeptide; and therefrom determining the presence or absence of a malignant disease associated with WT1 expression.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a comparison of the mouse (MO) and human (HU) WT1 protein sequences (SEQ ID NOS: 320 and 319 respectively).

Figure 2 is a Western blot illustrating the detection of WT1 specific antibodies in patients with hematological malignancy (AML). Lane 1 shows molecular weight markers; lane 2 shows a positive control (WT1 positive human leukemia cell line immunoprecipitated with a WT1 specific antibody); lane 3 shows a negative control (WT1 positive cell line immunoprecipitated with mouse sera); and lane 4 shows a WT1 positive cell line immunoprecipitated with sera of a patient with AML. For lanes 2-4, the immunoprecipitate was separated by gel electrophoresis and probed with a WT1 specific antibody.

Figure 3 is a Western blot illustrating the detection of a WT1 specific antibody response in B6 mice immunized with TrampC, a WT1 positive tumor cell line. Lane 1 shows molecular weight markers and lane 2 shows a WT1 specific positive control (N180, Santa Cruz Biotechnology, polypeptide spanning 180 amino acids of the N-terminal region of the WT1 protein, migrating on the Western blot at 52 kD) probed with sera of the immunized B6 mice.

Figure 4 is a Western blot illustrating the detection of WT1 specific antibodies in mice immunized with representative WT1 peptides. Lane 1 shows molecular weight markers and lane 2 shows a WT1 specific positive control (N180, Santa Cruz Biotechnology, polypeptide spanning 180 amino acids of the N-terminal region of the WT1 protein, migrating on the Western blot at 52 kD) probed with sera of non-immunized B6 mice. Lane 3 shows molecular weight markers and lane 4 shows the WT1 specific positive control probed with sera of the immunized B6 mice.

Figures 5A to 5C are graphs illustrating the stimulation of proliferative T cell responses in mice immunized with representative WT1 peptides. Thymidine incorporation assays were performed using one T cell line and two different clones, as indicated, and results were expressed as cpm. Controls indicated on the x axis were no antigen (No Ag) and B6/media; antigens used were p6-22 human (p1), p117-139 (p2) or p244-262 human (p3).

Figures 6A and 6B are graphs, illustrating the elicitation of WT1 peptide-specific CTL in mice immunized with WT1 peptides. Figure 6A illustrates the lysis of target cells by allogeneic cell lines and Figure 6B shows the lysis of peptide coated cell lines. In each case, the % lysis (as determined by standard chromium release assays) is shown at three indicated effector:target ratios. Results are provided for lymphoma cells (LSTRA and E10), as well as E10 + p235-243 (E10+P235).

Figures 7A-7D are graphs illustrating the elicitation of WT1 specific CTL, which kill WT1 positive tumor cell lines but do not kill WT1 negative cell lines, following vaccination of B6 mice with WT1 peptide P117. Figure 7A illustrates that T-cells of non-immunized B6 mice to not kill WT1 positive tumor cell lines. Figure 7B illustrates the lysis of the target cells by allogeneic cell lines. Figures 7C and 7D demonstrate the lysis of WT1 positive tumor cell lines, as compared to WT1 negative cell lines in two different experiments. In addition, Figures 7C and 7D show the lysis of peptide-coated cell lines (WT1 negative cell line E10 coated with the relevant WT1 peptide P117) In each case, the % lysis (as determined by standard chromium release assays) is shown at three indicated effector:target ratios. Results are provided for lymphoma cells (E10), prostate cancer cells (TrampC), a transformed fibroblast cell line (BLK SV40), as well as E10+p117.

Figures 8A and 8B present the results of TSITES Analysis of human WT1 (SEQ ID NO:319) for peptides that have the potential to elicit Th responses. Regions indicated by "A" are AMPHI midpoints of blocks, "R" indicates residues matching the Rothbard/'Taylor motif, "D" indicates residues matching the IAd motif, and 'd' indicates residues matching the IEd motif.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is generally directed to compositions and methods for the immunotherapy and diagnosis of malignant diseases. The compositions described herein may include WT1 polypeptides, WT1 polynucleotides, antigen-presenting cells (APC, *e.g.,* dendritic cells) that express a WT1 polypeptide, agents such as antibodies that bind to a WT1 polypeptide and/or immune system cells (*e.g.,* T cells) specific for WT1. WT1 Polypeptides of the present

invention generally comprise at least a portion of a Wilms Tumor gene product (WT1) or a variant thereof. Nucleic acid sequences of the subject invention generally comprise a DNA or RNA sequence that encodes all or a portion of such a polypeptide, or that is complementary to such a sequence. Antibodies are generally immune system proteins, or antigen-binding fragments thereof, that are capable of binding to a portion of a WT1 polypeptide. T cells that may be employed within such compositions are generally T cells (*e.g.*, $CD4^+$ and/or $CD8^+$) that are specific for a WT1 polypeptide. Certain methods described herein further employ antigen-presenting cells that express a WT1 polypeptide as provided herein.

The present invention is based on the discovery that an immune response raised against a Wilms Tumor (WT) gene product (*e.g.*, WT1) can provide prophylactic and/or therapeutic benefit for patients afflicted with malignant diseases characterized by increased WT1 gene expression. Such diseases include, but are not limited to, leukemias (*e.g.*, acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL) and childhood ALL), as well as many cancers such as lung, breast, thyroid and gastrointestinal cancers and melanomas. The WT1 gene was originally identified and isolated on the basis of a cytogenetic deletion at chromosome 11p13 in patients with Wilms' tumor (*see* Call et al., U.S. Patent No. 5,350,840). The gene consists of 10 exons and encodes a zinc finger transcription factor, and sequences of mouse and human WT1 proteins are provided in Figure 1 and SEQ ID NOs: 319 and 320.

WT1 POLYPEPTIDES

Within the context of the present invention, a WT1 polypeptide is a polypeptide that comprises at least an immunogenic portion of a native WT1 (*i.e.*, a WT1 protein expressed by an organism that is not genetically modified), or a variant thereof, as described herein. A WT1 polypeptide may be of any length, provided that it comprises at least an immunogenic portion of a native protein or a variant thereof. In other words, a WT1 polypeptide may be an oligopeptide (*i.e.*, consisting of a relatively small number of amino acid residues, such as 8-10 residues, joined by peptide bonds), a

full length WT1 protein (*e.g.,* present within a human or non-human animal, such as a mouse) or a polypeptide of intermediate size. Within certain embodiments, the use of WT1 polypeptides that contain a small number of consecutive amino acid residues of a native WT1 polypeptide is preferred. Such polypeptides are preferred for certain uses in which the generation of a T cell response is desired. For example, such a WT1 polypeptide may contain less than 23, preferably no more than 18, and more preferably no more than 15 consecutive amino acid residues, of a native WT1 polypeptide. Polypeptides comprising nine consecutive amino acid residues of a native WT1 polypeptide are generally suitable for such purposes. Additional sequences derived from the native protein and/or heterologous sequences may be present within any WT1 polypeptide, and such sequences may (but need not) possess further immunogenic or antigenic properties. Polypeptides as provided herein may further be associated (covalently or noncovalently) with other polypeptide or non-polypeptide compounds.

An "immunogenic portion," as used herein is a portion of a polypeptide that is recognized (*i.e.,* specifically bound) by a B-cell and/or T-cell surface antigen receptor. Certain preferred immunogenic portions bind to an MHC class I or class II molecule. As used herein, an immunogenic portion is said to "bind to" an MHC class I or class II molecule if such binding is detectable using any assay known in the art. For example, the ability of a polypeptide to bind to MHC class I may be evaluated indirectly by monitoring the ability to promote incorporation of ^{125}I labeled β2-microglobulin (β2m) into MHC class I/β2m/peptide heterotrimeric complexes (*see* Parker et al., *J. Immunol. 152*:163, 1994). Alternatively, functional peptide competition assays that are known in the art may be employed. Certain immunogenic portions have one or more of the sequences recited within one or more of Tables II - XIV. Representative immunogenic portions include, but are not limited to, RDLNALLPAVPSLGGGG (human WT1 residues 6-22; SEQ ID NO:1), PSQASSGQARMFPNAPYLPSCLE (human and mouse WT1 residues 117-139; SEQ ID NOs: 2 and 3 respectively), GATLKGVAAGSSSSVKWTE (human WT1 residues 244-262; SEQ ID NO:4), GATLKGVAA (human WT1 residues 244-252; SEQ ID NO:88), CMTWNQMNL (human and mouse WT1 residues 235-243; SEQ ID NOs: 49

and 258 respectively), SCLESQPTI (mouse WT1 residues 136-144; SEQ ID NO:296), SCLESQPAI (human WT1 residues 136-144; SEQ ID NO:198), NLYQMTSQL (human and mouse WT1 residues 225-233; SEQ ID NOs: 147 and 284 respectively); ALLPAVSSL (mouse WT1 residues 10-18; SEQ ID NO:255); or RMFPNAPYL (human and mouse WT1 residues 126-134; SEQ ID NOs: 185 and 293 respectively). Further immunogenic portions are provided herein, and others may generally be identified using well known techniques, such as those summarized in Paul, *Fundamental Immunology*, 3rd ed., 243-247 (Raven Press, 1993) and references cited therein. Representative techniques for identifying immunogenic portions include screening polypeptides for the ability to react with antigen-specific antisera and/or T-cell lines or clones. An immunogenic portion of a native WT1 polypeptide is a portion that reacts with such antisera and/or T-cells at a level that is not substantially less than the reactivity of the full length WT1 (*e.g.,* in an ELISA and/or T-cell reactivity assay). In other words, an immunogenic portion may react within such assays at a level that is similar to or greater than the reactivity of the full length polypeptide. Such screens may generally be performed using methods well known to those of ordinary skill in the art, such as those described in Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988.

Alternatively, immunogenic portions may be identified using computer analysis, such as the Tsites program (*see* Rothbard and Taylor, *EMBO J.* 7:93-100, 1988; Deavin et al., *Mol. Immunol.* 33:145-155, 1996), which searches for peptide motifs that have the potential to elicit Th responses. CTL peptides with motifs appropriate for binding to murine and human class I or class II MHC may be identified according to BIMAS (Parker et al., *J. Immunol.* 152:163, 1994) and other HLA peptide binding prediction analyses. To confirm immunogenicity, a peptide may be tested using an HLA A2 transgenic mouse model and/or an *in vitro* stimulation assay using dendritic cells, fibroblasts or peripheral blood cells.

As noted above, a composition may comprise a variant of a native WT1 protein. A polypeptide "variant," as used herein, is a polypeptide that differs from a native polypeptide in one or more substitutions, deletions, additions and/or insertions,

such that the immunogenicity of the polypeptide is retained (*i.e.*, the ability of the variant to react with antigen-specific antisera and/or T-cell lines or clones is not substantially diminished relative to the native polypeptide). In other words, the ability of a variant to react with antigen-specific antisera and/or T-cell lines or clones may be enhanced or unchanged, relative to the native polypeptide, or may be diminished by less than 50%, and preferably less than 20%, relative to the native polypeptide. Such variants may generally be identified by modifying one of the above polypeptide sequences and evaluating the reactivity of the modified polypeptide with antisera and/or T-cells as described herein. It has been found, within the context of the present invention, that a relatively small number of substitutions (*e.g.*, 1 to 3) within an immunogenic portion of a WT1 polypeptide may serve to enhance the ability of the polypeptide to elicit an immune response. Suitable substitutions may generally be identified by using computer programs, as described above, and the effect confirmed based on the reactivity of the modified polypeptide with antisera and/or T-cells as described herein. Accordingly, within certain preferred embodiments, a WT1 polypeptide comprises a variant in which 1 to 3 amino acid resides within an immunogenic portion are substituted such that the ability to react with antigen-specific antisera and/or T-cell lines or clones is statistically greater than that for the unmodified polypeptide. Such substitutions are preferably located within an MHC binding site of the polypeptide, which may be identified as described above. Preferred substitutions allow increased binding to MHC class I or class II molecules.

Certain variants contain conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Amino acid substitutions may generally be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values

include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also, or alternatively, contain nonconservative changes. Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the immunogenicity, secondary structure and hydropathic nature of the polypeptide.

As noted above, WT1 polypeptides may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. A polypeptide may also, or alternatively, be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (*e.g.,* poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

WT1 polypeptides may be prepared using any of a variety of well known techniques. Recombinant polypeptides encoded by a WT1 polynucleotide as described herein may be readily prepared from the polynucleotide. In general, any of a variety of expression vectors known to those of ordinary skill in the art may be employed to express recombinant WT1 polypeptides. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule that encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast and higher eukaryotic cells. Preferably, the host cells employed are *E. coli*, yeast or a mammalian cell line such as COS or CHO. Supernatants from suitable host/vector systems which secrete recombinant protein or polypeptide into culture media may be first concentrated using a commercially available filter. The concentrate may then be applied to a suitable purification matrix such as an affinity matrix or an ion exchange resin. Finally, one or more reverse phase HPLC steps can be employed to further purify a recombinant polypeptide. Such techniques may be used to prepare native polypeptides or variants thereof. For example,

polynucleotides that encode a variant of a native polypeptide may generally be prepared using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis, and sections of the DNA sequence may be removed to permit preparation of truncated polypeptides.

Certain portions and other variants may also be generated by synthetic means, using techniques well known to those of ordinary skill in the art. For example, polypeptides having fewer than about 500 amino acids, preferably fewer than about 100 amino acids, and more preferably fewer than about 50 amino acids, may be synthesized. Polypeptides may be synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. *See* Merrifield, *J. Am. Chem. Soc.* *85*:2149-2146, 1963. Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Applied BioSystems, Inc. (Foster City, CA), and may be operated according to the manufacturer's instructions.

In general, polypeptides and polynucleotides as described herein are isolated. An "isolated" polypeptide or polynucleotide is one that is removed from its original environment. For example, a naturally-occurring protein is isolated if it is separated from some or all of the coexisting materials in the natural system. Preferably, such polypeptides are at least about 90% pure, more preferably at least about 95% pure and most preferably at least about 99% pure. A polynucleotide is considered to be isolated if, for example, it is cloned into a vector that is not a part of the natural environment.

Within further aspects, the present invention provides mimetics of WT1 polypeptides. Such mimetics may comprise amino acids linked to one or more amino acid mimetics (*i.e.*, one or more amino acids within the WT1 protein may be replaced by an amino acid mimetic) or may be entirely nonpeptide mimetics. An amino acid mimetic is a compound that is conformationally similar to an amino acid such that it can be substituted for an amino acid within a WT1 polypeptide without substantially diminishing the ability to react with antigen-specific antisera and/or T cell lines or clones. A nonpeptide mimetic is a compound that does not contain amino acids, and

that has an overall conformation that is similar to a WT1 polypeptide such that the ability of the mimetic to react with WT1-specific antisera and/or T cell lines or clones is not substantially diminished relative to the ability of a WT1 polypeptide. Such mimetics may be designed based on standard techniques (*e.g.,* nuclear magnetic resonance and computational techniques) that evaluate the three dimensional structure of a peptide sequence. Mimetics may be designed where one or more of the side chain functionalities of the WT1 polypeptide are replaced by groups that do not necessarily have the same size or volume, but have similar chemical and/or physical properties which produce similar biological responses. It should be understood that, within embodiments described herein, a mimetic may be substituted for a WT1 polypeptide.

WT1 POLYNUCLEOTIDES

Any polynucleotide that encodes a WT1 polypeptide as described herein is a WT1 polynucleotide encompassed by the present invention. Such polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide of the present invention, and a polynucleotide may, but need not, be linked to other molecules and/or support materials.

WT1 polynucleotides may encode a native WT1 protein, or may encode a variant of WT1 as described herein. Polynucleotide variants may contain one or more substitutions, additions, deletions and/or insertions such that the immunogenicity of the encoded polypeptide is not diminished, relative to a native WT1 protein. The effect on the immunogenicity of the encoded polypeptide may generally be assessed as described herein. Preferred variants contain nucleotide substitutions, deletions, insertions and/or additions at no more than 20%, preferably at no more than 10%, of the nucleotide positions that encode an immunogenic portion of a native WT1 sequence. Certain variants are substantially homologous to a native gene, or a portion thereof. Such polynucleotide variants are capable of hybridizing under moderately stringent conditions to a naturally occurring DNA sequence encoding a WT1 polypeptide (or a

complementary sequence). Suitable moderately stringent conditions include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS). Such hybridizing DNA sequences are also within the scope of this invention.

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a WT1 polypeptide. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated by the present invention.

Once an immunogenic portion of WT1 is identified, as described above, a WT1 polynucleotide may be prepared using any of a variety of techniques. For example, a WT1 polynucleotide may be amplified from cDNA prepared from cells that express WT1. Such polynucleotides may be amplified via polymerase chain reaction (PCR). For this approach, sequence-specific primers may be designed based on the sequence of the immunogenic portion and may be purchased or synthesized. For example, suitable primers for PCR amplification of a human WT1 gene include: first step - P118: 1434-1414: 5' GAG AGT CAG ACT TGA AAG CAGT 3' (SEQ ID NO:5) and P135: 5' CTG AGC CTC AGC AAA TGG GC 3' (SEQ ID NO:6); second step - P136: 5' GAG CAT GCA TGG GCT CCG ACG TGC GGG 3' (SEQ ID NO:7) and P137: 5' GGG GTA CCC ACT GAA CGG TCC CCG A 3' (SEQ ID NO:8). Primers for PCR amplification of a mouse WT1 gene include: first step - P138: 5' TCC GAG CCG CAC CTC ATG 3' (SEQ ID NO:9) and P139: 5' GCC TGG GAT GCT GGA CTG 3' (SEQ ID NO:10), second step - P140: 5' GAG CAT GCG ATG GGT TCC GAC GTG CGG 3' (SEQ ID NO:11) and P141: 5' GGG GTA CCT CAA AGC GCC ACG TGG AGT TT 3' (SEQ ID NO:12).

An amplified portion may then be used to isolate a full length gene from a human genomic DNA library or from a suitable cDNA library, using well known techniques. Alternatively, a full length gene can be constructed from multiple PCR

fragments. WT1 polynucleotides may also be prepared by synthesizing oligonucleotide components, and ligating components together to generate the complete polynucleotide.

WT1 polynucleotides may also be synthesized by any method known in the art, including chemical synthesis (*e.g.,* solid phase phosphoramidite chemical synthesis). Modifications in a polynucleotide sequence may also be introduced using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis (*see* Adelman et al., *DNA 2*:183, 1983). Alternatively, RNA molecules may be generated by *in vitro* or *in vivo* transcription of DNA sequences encoding a WT1 polypeptide, provided that the DNA is incorporated into a vector with a suitable RNA polymerase promoter (such as T7 or SP6). Certain portions may be used to prepare an encoded polypeptide, as described herein. In addition, or alternatively, a portion may be administered to a patient such that the encoded polypeptide is generated *in vivo* (*e.g.,* by transfecting antigen-presenting cells such as dendritic cells with a cDNA construct encoding a WT1 polypeptide, and administering the transfected cells to the patient).

Polynucleotides that encode a WT1 polypeptide may generally be used for production of the polypeptide, *in vitro* or *in vivo*. WT1 polynucleotides that are complementary to a coding sequence (*i.e.,* antisense polynucleotides) may also be used as a probe or to inhibit WT1 expression. cDNA constructs that can be transcribed into antisense RNA may also be introduced into cells of tissues to facilitate the production of antisense RNA.

Any polynucleotide may be further modified to increase stability *in vivo*. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends; the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages in the backbone; and/or the inclusion of nontraditional bases such as inosine, queosine and wybutosine, as well as acetyl- methyl-, thio- and other modified forms of adenine, cytidine, guanine, thymine and uridine.

Nucleotide sequences as described herein may be joined to a variety of other nucleotide sequences using established recombinant DNA techniques. For example, a polynucleotide may be cloned into any of a variety of cloning vectors,

including plasmids, phagemids, lambda phage derivatives and cosmids. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors and sequencing vectors. In general, a vector will contain an origin of replication functional in at least one organism, convenient restriction endonuclease sites and one or more selectable markers. Other elements will depend upon the desired use, and will be apparent to those of ordinary skill in the art.

Within certain embodiments, polynucleotides may be formulated so as to permit entry into a cell of a mammal, and expression therein. Such formulations are particularly useful for therapeutic purposes, as described below. Those of ordinary skill in the art will appreciate that there are many ways to achieve expression of a polynucleotide in a target cell, and any suitable method may be employed. For example, a polynucleotide may be incorporated into a viral vector such as, but not limited to, adenovirus, adeno-associated virus, retrovirus, or vaccinia or other pox virus (*e.g.,* avian pox virus). Techniques for incorporating DNA into such vectors are well known to those of ordinary skill in the art. A retroviral vector may additionally transfer or incorporate a gene for a selectable marker (to aid in the identification or selection of transduced cells) and/or a targeting moiety, such as a gene that encodes a ligand for a receptor on a specific target cell, to render the vector target specific. Targeting may also be accomplished using an antibody, by methods known to those of ordinary skill in the art. cDNA constructs within such a vector may be used, for example, to transfect human or animal cell lines for use in establishing WT1 positive tumor models which may be used to perform tumor protection and adoptive immunotherapy experiments to demonstrate tumor or leukemia-growth inhibition or lysis of such cells.

Other therapeutic formulations for polynucleotides include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (*i.e.,* an artificial membrane vesicle). The preparation and use of such systems is well known in the art.

ANTIBODIES AND FRAGMENTS THEREOF

The present invention further provides binding agents, such as antibodies and antigen-binding fragments thereof, that specifically bind to a WT1 polypeptide. As used herein, an agent is said to "specifically bind" to a WT1 polypeptide if it reacts at a detectable level (within, for example, an ELISA) with a WT1 polypeptide, and does not react detectably with unrelated proteins under similar conditions. As used herein, "binding" refers to a noncovalent association between two separate molecules such that a "complex" is formed. The ability to bind may be evaluated by, for example, determining a binding constant for the formation of the complex. The binding constant is the value obtained when the concentration of the complex is divided by the product of the component concentrations. In general, two compounds are said to "bind, in the context of the present invention, when the binding constant for complex formation exceeds about 10^3 L/mol. The binding constant maybe determined using methods well known in the art.

Any agent that satisfies the above requirements may be a binding agent. In a preferred embodiment, a binding agent is an antibody or an antigen-binding fragment thereof. Certain antibodies are commercially available from, for example, Santa Cruz Biotechnology (Santa Cruz, CA). Alternatively, antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art. *See, e.g.,* Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In general, antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies as described herein, or via transfection of antibody genes into suitable bacterial or mammalian cell hosts, in order to allow for the production of recombinant antibodies. In one technique, an immunogen comprising the polypeptide is initially injected into any of a wide variety of mammals (*e.g.,* mice, rats, rabbits, sheep or goats). In this step, the polypeptides of this invention may serve as the immunogen without modification. Alternatively, particularly for relatively short polypeptides, a superior immune response may be elicited if the polypeptide is joined to a carrier protein, such as bovine serum albumin or keyhole limpet hemocyanin. The immunogen is injected into the animal host, preferably according to a predetermined

schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Monoclonal antibodies specific for the antigenic polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol. 6*:511-519, 1976, and improvements thereto. Briefly, these methods involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and their culture supernatants tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. The polypeptides of this invention may be used in the purification process in, for example, an affinity chromatography step.

Within certain embodiments, the use of antigen-binding fragments of antibodies may be preferred. Such fragments include Fab fragments, which may be

prepared using standard techniques. Briefly, immunoglobulins may be purified from rabbit serum by affinity chromatography on Protein A bead columns (Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988) and digested by papain to yield Fab and Fc fragments. The Fab and Fc fragments may be separated by affinity chromatography on protein A bead columns.

Monoclonal antibodies and fragments thereof may be coupled to one or more therapeutic agents. Suitable agents in this regard include radioactive tracers and chemotherapeutic agents, which may be used, for example, to purge autologous bone marrow *in vitro*). Representative therapeutic agents include radionuclides, differentiation inducers, drugs, toxins, and derivatives thereof. Preferred radionuclides include ^{90}Y, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, ^{211}At, and ^{212}Bi. Preferred drugs include methotrexate, and pyrimidine and purine analogs. Preferred differentiation inducers include phorbol esters and butyric acid. Preferred toxins include ricin, abrin, diptheria toxin, cholera toxin, gelonin, Pseudomonas exotoxin, Shigella toxin, and pokeweed antiviral protein. For diagnostic purposes, coupling of radioactive agents may be used to facilitate tracing of metastases or to determine the location of WT1-positive tumors.

A therapeutic agent may be coupled (*e.g.*, covalently bonded) to a suitable monoclonal antibody either directly or indirectly (*e.g.*, via a linker group). A direct reaction between an agent and an antibody is possible when each possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl group, on one may be capable of reacting with a carbonyl-containing group, such as an anhydride or an acid halide, or with an alkyl group containing a good leaving group (*e.g.*, a halide) on the other.

Alternatively, it may be desirable to couple a therapeutic agent and an antibody via a linker group. A linker group can function as a spacer to distance an antibody from an agent in order to avoid interference with binding capabilities. A linker group can also serve to increase the chemical reactivity of a substituent on an agent or an antibody, and thus increase the coupling efficiency. An increase in chemical reactivity may also facilitate the use of agents, or functional groups on agents, which otherwise would not be possible.

It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional (such as those described in the catalog of the Pierce Chemical Co., Rockford, IL), may be employed as the linker group. Coupling may be effected, for example, through amino groups, carboxyl groups, sulfhydryl groups or oxidized carbohydrate residues. There are numerous references describing such methodology, *e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.

Where a therapeutic agent is more potent when free from the antibody portion of the immunoconjugates of the present invention, it may be desirable to use a linker group which is cleavable during or upon internalization into a cell. A number of different cleavable linker groups have been described. The mechanisms for the intracellular release of an agent from these linker groups include cleavage by reduction of a disulfide bond (*e.g.*, U.S. Patent No. 4,489,710, to Spitler), by irradiation of a photolabile bond (*e.g.*, U.S. Patent No. 4,625,014, to Senter et al.), by hydrolysis of derivatized amino acid side chains (*e.g.*, U.S. Patent No. 4,638,045, to Kohn et al.), by serum complement-mediated hydrolysis (*e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.), and acid-catalyzed hydrolysis (*e.g.*, U.S. Patent No. 4,569,789, to Blattler et al.).

It may be desirable to couple more than one agent to an antibody. In one embodiment, multiple molecules of an agent are coupled to one antibody molecule. In another embodiment, more than one type of agent may be coupled to one antibody. Regardless of the particular embodiment, immunoconjugates with more than one agent may be prepared in a variety of ways. For example, more than one agent may be coupled directly to an antibody molecule, or linkers which provide multiple sites for attachment can be used. Alternatively, a carrier can be used. A carrier may bear the agents in a variety of ways, including covalent bonding either directly or via a linker group. Suitable carriers include proteins such as albumins (*e.g.*, U.S. Patent No. 4,507,234, to Kato et al.), peptides and polysaccharides such as aminodextran (*e.g.*, U.S. Patent No. 4,699,784, to Shih et al.). A carrier may also bear an agent by noncovalent bonding or by encapsulation, such as within a liposome vesicle (*e.g.*, U.S. Patent Nos. 4,429,008 and 4,873,088). Carriers specific for radionuclide agents include radiohalogenated small molecules and chelating compounds. For example, U.S. Patent

No. 4,735,792 discloses representative radiohalogenated small molecules and their synthesis. A radionuclide chelate may be formed from chelating compounds that include those containing nitrogen and sulfur atoms as the donor atoms for binding the metal, or metal oxide, radionuclide. For example, U.S. Patent No. 4,673,562, to Davison et al. discloses representative chelating compounds and their synthesis.

A variety of routes of administration for the antibodies and immunoconjugates may be used. Typically, administration will be intravenous, intramuscular, subcutaneous or in the bed of a resected tumor. It will be evident that the precise dose of the antibody/immunoconjugate will vary depending upon the antibody used, the antigen density on the tumor, and the rate of clearance of the antibody.

Also provided herein are anti-idiotypic antibodies that mimic an immunogenic portion of WT1. Such antibodies may be raised against an antibody, or antigen-binding fragment thereof, that specifically binds to an immunogenic portion of WT1, using well known techniques. Anti-idiotypic antibodies that mimic an immunogenic portion of WT1 are those antibodies that bind to an antibody, or antigen-binding fragment thereof, that specifically binds to an immunogenic portion of WT1, as described herein.

T CELLS

Immunotherapeutic compositions may also, or alternatively, comprise T cells specific for WT1. Such cells may generally be prepared *in vitro* or *ex vivo*, using standard procedures. For example, T cells may be present within (or isolated from) bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood of a mammal, such as a patient, using a commercially available cell separation system, such as the CEPRATE™ system, available from CellPro Inc., Bothell WA (see also U.S. Patent No. 5,240,856; U.S. Patent No. 5,215,926; WO 89/06280; WO 91/16116 and WO 92/07243). Alternatively, T cells may be derived from related or unrelated humans, non-human animals, cell lines or cultures.

T cells may be stimulated with WT1 polypeptide, polynucleotide encoding a WT1 polypeptide and/or an antigen presenting cell (APC) that expresses a

WT1 polypeptide. Such stimulation is performed under conditions and for a time sufficient to permit the generation of T cells that are specific for the WT1 polypeptide. Preferably, a WT1 polypeptide or polynucleotide is present within a delivery vehicle, such as a microsphere, to facilitate the generation of antigen-specific T cells. Briefly, T cells, which may be isolated from a patient or a related or unrelated donor by routine techniques (such as by Ficoll/Hypaque density gradient centrifugation of peripheral blood lymphocytes), are incubated with WT1 polypeptide. For example, T cells may be incubated *in vitro* for 2-9 days (typically 4 days) at 37°C with WT1 polypeptide (*e.g.*, 5 to 25 μg/ml) or cells synthesizing a comparable amount of WT1 polypeptide. It may be desirable to incubate a separate aliquot of a T cell sample in the absence of WT1 polypeptide to serve as a control.

T cells are considered to be specific for a WT1 polypeptide if the T cells kill target cells coated with a WT1 polypeptide or expressing a gene encoding such a polypeptide. T cell specificity may be evaluated using any of a variety of standard techniques. For example, within a chromium release assay or proliferation assay, a stimulation index of more than two fold increase in lysis and/or proliferation, compared to negative controls, indicates T cell specificity. Such assays may be performed, for example, as described in Chen et al., *Cancer Res. 54*:1065-1070, 1994. Alternatively, detection of the proliferation of T cells may be accomplished by a variety of known techniques. For example, T cell proliferation can be detected by measuring an increased rate of DNA synthesis (*e.g.*, by pulse-labeling cultures of T cells with tritiated thymidine and measuring the amount of tritiated thymidine incorporated into DNA). Other ways to detect T cell proliferation include measuring increases in interleukin-2 (IL-2) production, Ca^{2+} flux, or dye uptake, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium. Alternatively, synthesis of lymphokines (such as interferon-gamma) can be measured or the relative number of T cells that can respond to a WT1 polypeptide may be quantified. Contact with a WT1 polypeptide (200 ng/ml - 100 μg/ml, preferably 100 ng/ml - 25 μg/ml) for 3 - 7 days should result in at least a two fold increase in proliferation of the T cells and/or contact as described above for 2-3 hours should result in activation of the T cells, as measured using standard cytokine

assays in which a two fold increase in the level of cytokine release (*e.g.*, TNF or IFN-γ) is indicative of T cell activation (*see* Coligan et al., Current Protocols in Immunology, vol. 1, Wiley Interscience (Greene 1998). WT1 specific T cells may be expanded using standard techniques. Within preferred embodiments, the T cells are derived from a patient or a related or unrelated donor and are administered to the patient following stimulation and expansion.

T cells that have been activated in response to a WT1 polypeptide, polynucleotide or WT1-expressing APC may be $CD4^+$ and/or $CD8^+$. Specific activation of $CD4^+$ or $CD8^+$ T cells may be detected in a variety of ways. Methods for detecting specific T cell activation include detecting the proliferation of T cells, the production of cytokines (*e.g.*, lymphokines), or the generation of cytolytic activity (*i.e.*, generation of cytotoxic T cells specific for WT1). For $CD4^+$ T cells, a preferred method for detecting specific T cell activation is the detection of the proliferation of T cells. For $CD8^+$ T cells, a preferred method for detecting specific T cell activation is the detection of the generation of cytolytic activity.

For therapeutic purposes, $CD4^+$ or $CD8^+$ T cells that proliferate in response to the WT1 polypeptide, polynucleotide or APC can be expanded in number either *in vitro* or *in vivo*. Proliferation of such T cells *in vitro* may be accomplished in a variety of ways. For example, the T cells can be re-exposed to WT1 polypeptide, with or without the addition of T cell growth factors, such as interleukin-2, and/or stimulator cells that synthesize a WT1 polypeptide. The addition of stimulator cells is preferred where generating $CD8^+$ T cell responses. T cells can be grown to large numbers *in vitro* with retention of specificity in response to intermittent restimulation with WT1 polypeptide. Briefly, for the primary *in vitro* stimulation (IVS), large numbers of lymphocytes (*e.g.*, greater than 4×10^7) may be placed in flasks with media containing human serum. WT1 polypeptide (*e.g.*, peptide at 10 μg/ml) may be added directly, along with tetanus toxoid (*e.g.*, 5 μg/ml). The flasks may then be incubated (*e.g.*, 37°C for 7 days). For a second IVS, T cells are then harvested and placed in new flasks with $2\text{-}3 \times 10^7$ irradiated peripheral blood mononuclear cells. WT1 polypeptide (*e.g.*, 10 μg/ml) is added directly. The flasks are incubated at 37°C for 7 days. On day 2 and

day 4 after the second IVS, 2-5 units of interleukin-2 (IL-2) may be added. For a third IVS, the T cells may be placed in wells and stimulated with the individual's own EBV transformed B cells coated with the peptide. IL-2 may be added on days 2 and 4 of each cycle. As soon as the cells are shown to be specific cytotoxic T cells, they may be expanded using a 10 day stimulation cycle with higher IL-2 (20 units) on days 2, 4 and 6.

Alternatively, one or more T cells that proliferate in the presence of WT1 polypeptide can be expanded in number by cloning. Methods for cloning cells are well known in the art, and include limiting dilution. Responder T cells may be purified from the peripheral blood of sensitized patients by density gradient centrifugation and sheep red cell rosetting and established in culture by stimulating with the nominal antigen in the presence of irradiated autologous filler cells. In order to generate $CD4^+$ T cell lines, WT1 polypeptide is used as the antigenic stimulus and autologous peripheral blood lymphocytes (PBL) or lymphoblastoid cell lines (LCL) immortalized by infection with Epstein Barr virus are used as antigen presenting cells. In order to generate $CD8^+$ T cell lines, autologous antigen-presenting cells transfected with an expression vector which produces WT1 polypeptide may be used as stimulator cells. Established T cell lines may be cloned 2-4 days following antigen stimulation by plating stimulated T cells at a frequency of 0.5 cells per well in 96-well flat-bottom plates with 1×10^6 irradiated PBL or LCL cells and recombinant interleukin-2 (rIL2) (50 U/ml). Wells with established clonal growth may be identified at approximately 2-3 weeks after initial plating and restimulated with appropriate antigen in the presence of autologous antigen-presenting cells, then subsequently expanded by the addition of low doses of rIL2 (10 U/ml) 2-3 days following antigen stimulation. T cell clones may be maintained in 24-well plates by periodic restimulation with antigen and rIL2 approximately every two weeks.

Within certain embodiments, allogeneic T-cells may be primed (*i.e.*, sensitized to WT1) *in vivo* and/or *in vitro*. Such priming may be achieved by contacting T cells with a WT1 polypeptide, a polynucleotide encoding such a polypeptide or a cell producing such a polypeptide under conditions and for a time sufficient to permit the

priming of T cells. In general, T cells are considered to be primed if, for example, contact with a WT1 polypeptide results in proliferation and/or activation of the T cells, as measured by standard proliferation, chromium release and/or cytokine release assays as described herein. A stimulation index of more than two fold increase in proliferation or lysis, and more than three fold increase in the level of cytokine, compared to negative controls, indicates T-cell specificity. Cells primed *in vitro* may be employed, for example, within a bone marrow transplantation or as donor lymphocyte infusion.

PHARMACEUTICAL COMPOSITIONS AND VACCINES

Within certain aspects, polypeptides, polynucleotides, antibodies and/or T cells may be incorporated into pharmaceutical compositions or vaccines. Alternatively, a pharmaceutical composition may comprise an antigen-presenting cell (*e.g.,* a dendritic cell) transfected with a WT1 polynucleotide such that the antigen presenting cell expresses a WT1 polypeptide. Pharmaceutical compositions comprise one or more such compounds or cells and a physiologically acceptable carrier or excipient. Certain vaccines may comprise one or more such compounds or cells and a non-specific immune response enhancer, such as an adjuvant or a liposome (into which the compound is incorporated). Pharmaceutical compositions and vaccines may additionally contain a delivery system, such as biodegradable microspheres which are disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109. Pharmaceutical compositions and vaccines within the scope of the present invention may also contain other compounds, which may be biologically active or inactive.

Within certain embodiments, pharmaceutical compositions and vaccines are designed to elicit T cell responses specific for a WT1 polypeptide in a patient, such as a human. In general, T cell responses may be favored through the use of relatively short polypeptides (*e.g.,* comprising less than 23 consecutive amino acid residues of a native WT1 polypeptide, preferably 4-16 consecutive residues, more preferably 8-16 consecutive residues and still more preferably 8-10 consecutive residues. Alternatively, or in addition, a vaccine may comprise a non-specific immune response enhancer that preferentially enhances a T cell response. In other words, the immune response

enhancer may enhance the level of a T cell response to a WT1 polypeptide by an amount that is proportionally greater than the amount by which an antibody response is enhanced. For example, when compared to a standard oil based adjuvant, such as CFA, an immune response enhancer that preferentially enhances a T cell response may enhance a proliferative T cell response by at least two fold, a lytic response by at least 10%, and/or T cell activation by at least two fold compared to WT1-megative control cell lines, while not detectably enhancing an antibody response. The amount by which a T cell or antibody response to a WT1 polypeptide is enhanced may generally be determined using any representative technique known in the art, such as the techniques provided herein.

A pharmaceutical composition or vaccine may contain DNA encoding one or more of the polypeptides as described above, such that the polypeptide is generated *in situ*. As noted above, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacterial and viral expression systems and mammalian expression systems. Appropriate nucleic acid expression systems contain the necessary DNA, cDNA or RNA sequences for expression in the patient (such as a suitable promoter and terminating signal). Bacterial delivery systems involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an immunogenic portion of the polypeptide on its cell surface. In a preferred embodiment, the DNA may be introduced using a viral expression system (*e.g.*, vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in Ulmer et al., *Science 259*:1745-1749, 1993 and reviewed by Cohen, *Science 259*:1691-1692, 1993. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

As noted above, a pharmaceutical composition or vaccine may comprise an antigen-presenting cell that expresses a WT1 polypeptide. For therapeutic purposes,

as described herein, the antigen presenting cell is preferably an autologous dendritic cell. Such cells may be prepared and transfected using standard techniques, such as those described by Reeves et al., *Cancer Res. 56*:5672-5677, 1996; Tuting et al., *J. Immunol. 160*:1139-1147, 1998; and Nair et al., *Nature Biotechnol. 16*:364-369, 1998). Expression of a WT1 polypeptide on the surface of an antigen-presenting cell may be confirmed by *in vitro* stimulation and standard proliferation as well as chromium release assays, as described herein.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (*e.g.,* polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention. For certain topical applications, formulation as a cream or lotion, using well known components, is preferred.

Such compositions may also comprise buffers (*e.g.,* neutral buffered saline or phosphate buffered saline), carbohydrates (*e.g.,* glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione, adjuvants (*e.g.*, aluminum hydroxide) and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate. Compounds may also be encapsulated within liposomes using well known technology.

Any of a variety of non-specific immune response enhancers, such as adjuvants, may be employed in the vaccines of this invention. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum

hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bortadella pertussis* or *Mycobacterium tuberculosis* derived proteins. Suitable non-specific immune response enhancers include alum-based adjuvants (*e.g.,* Alhydrogel, Rehydragel, aluminum phosphate, Algammulin, aluminum hydroxide); oil based adjuvants (Freund's adjuvant (FA), Specol, RIBI, TiterMax, Montanide ISA50 or Seppic MONTANIDE ISA 720; cytokines (*e.g.,* GM-CSF or Flat3-ligand); microspheres; nonionic block copolymer-based adjuvants; dimethyl dioctadecyl ammoniumbromide (DDA) based adjuvants AS-1, AS-2 (Smith Kline Beecham); Ribi Adjuvant system based adjuvants; QS21 (Aquila); saponin based adjuvants (crude saponin, the saponin Quil A); muramyl dipeptide (MDP) based adjuvants such as SAF (Syntex adjuvant in its microfluidized form (SAF-m)); dimethyl-dioctadecyl ammonium bromide (DDA); human complement based adjuvants *m. vaccae* and derivatives; immune stimulating complex (iscom) based adjuvants; inactivated toxins; and attenuated infectious agents (such as *M. tuberculosis*).

As noted above, within certain embodiments, immune response enhancers are chosen for their ability to preferentially elicit or enhance a T cell response (*e.g.,* $CD4^+$ and/or $CD8^+$) to a WT1 polypeptide. Such immune response enhancers are well known in the art, and include (but are not limited to) Montanide ISA50, Seppic MONTANIDE ISA 720, cytokines (*e.g.,* GM-CSF, Flat3-ligand), microspheres, dimethyl dioctadecyl ammoniumbromide (DDA) based adjuvants, AS-1 (Smith Kline Beecham), AS-2 (Smith Kline Beecham), Ribi Adjuvant system based adjuvants, QS21 (Aquila), saponin based adjuvants (crude saponin, the saponin Quil A), Syntex adjuvant in its microfluidized form (SAF-m), MV; ddMV (Genesis), immune stimulating complex (iscom) based adjuvants and inactivated toxins.

The compositions and vaccines described herein may be administered as part of a sustained release formulation (*i.e.,* a formulation such as a capsule or sponge that effects a slow release of compound following administration). Such formulations may generally be prepared using well known technology and administered by, for example, oral, rectal or subcutaneous implantation, or by implantation at the desired target site. Sustained-release formulations may contain a polypeptide, polynucleotide,

antibody or cell dispersed in a carrier matrix and/or contained within a reservoir surrounded by a rate controlling membrane. Carriers for use within such formulations are biocompatible, and may also be biodegradable; preferably the formulation provides a relatively constant level of active component release. The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

THERAPY OF MALIGNANT DISEASES

In further aspects of the present invention, the compositions and vaccines described herein may be used to inhibit the development of malignant diseases (*e.g.*, progressive or metastatic diseases or diseases characterized by small tumor burden such as minimal residual disease). In general, such methods may be used to prevent, delay or treat a disease associated with WT1 expression. In other words, therapeutic methods provided herein may be used to treat an existing WT1-associated disease, or may be used to prevent or delay the onset of such a disease in a patient who is free of disease or who is afflicted with a disease that is not yet associated with WT1 expression.

As used herein, a disease is "associated with WT1 expression" if diseased cells (*e.g.*, tumor cells) at some time during the course of the disease generate detectably higher levels of a WT1 polypeptide than normal cells of the same tissue. Association of WT1 expression with a malignant disease does not require that WT1 be present on a tumor. For example, overexpression of WT1 may be involved with initiation of a tumor, but the protein expression may subsequently be lost. Alternatively, a malignant disease that is not characterized by an increase in WT1 expression may, at a later time, progress to a disease that is characterized by increased WT1 expression. Accordingly, any malignant disease in which diseased cells formerly expressed, currently express or are expected to subsequently express increased levels of WT1 is considered to be "associated with WT1 expression."

Immunotherapy may be performed using any of a variety of techniques, in which compounds or cells provided herein function to remove WT1-expressing cells

from a patient. Such removal may take place as a result of enhancing or inducing an immune response in a patient specific for WT1 or a cell expressing WT1. Alternatively, WT1-expressing cells may be removed *ex vivo* (*e.g.*, by treatment of autologous bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood). Fractions of bone marrow or peripheral blood may be obtained using any standard technique in the art.

Within such methods, pharmaceutical compositions and vaccines may be administered to a patient. As used herein, a "patient" refers to any warm-blooded animal, preferably a human. A patient may or may not be afflicted with a malignant disease. Accordingly, the above pharmaceutical compositions and vaccines may be used to prevent the onset of a disease (*i.e.*, prophylactically) or to treat a patient afflicted with a disease (*e.g.*, to prevent or delay progression and/or metastasis of an existing disease). A patient afflicted with a disease may have a minimal residual disease (*e.g.*, a low tumor burden in a leukemia patient in complete or partial remission or a cancer patient following reduction of the tumor burden after surgery radiotherapy and/or chemotherapy). Such a patient may be immunized to inhibit a relapse (*i.e.*, prevent or delay the relapse, or decrease the severity of a relapse). Within certain preferred embodiments, the patient is afflicted with a leukemia (*e.g.*, AML, CML, ALL or childhood ALL), a myelodysplastic syndrome (MDS) or a cancer (*e.g.*, gastrointestinal, lung, thyroid or breast cancer or a melanoma), where the cancer or leukemia is WT1 positive (*i.e.*, reacts detectably with an anti-WT1 antibody, as provided herein or expresses WT1 mRNA at a level detectable by RT-PCR, as described herein) or suffers from an autoimmune disease directed against WT1-expressing cells.

The compositions provided herein may be used alone or in combination with conventional therapeutic regimens such as surgery, irradiation, chemotherapy and/or bone marrow transplantation (autologous, syngeneic, allogeneic or unrelated). As discussed in greater detail below, binding agents and T cells as provided herein may be used for purging of autologous stem cells. Such purging may be beneficial prior to, for example, bone marrow transplantation or transfusion of blood or components thereof. Binding agents, T cells, antigen presenting cells (APC) and compositions

provided herein may further be used for expanding and stimulating (or priming) autologous, allogeneic, syngeneic or unrelated WT1-specific T-cells *in vitro* and/or *in vivo*. Such WT1-specific T cells may be used, for example, within donor lymphocyte infusions.

Routes and frequency of administration, as well as dosage, will vary from individual to individual, and may be readily established using standard techniques. In general, the pharmaceutical compositions and vaccines may be administered by injection (*e.g.*, intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (*e.g.*, by aspiration) or orally. In some tumors, pharmaceutical compositions or vaccines may be administered locally (by, for example, rectocoloscopy, gastroscopy, videoendoscopy, angiography or other methods known in the art). Preferably, between 1 and 10 doses may be administered over a 52 week period. Preferably, 6 doses are administered, at intervals of 1 month, and booster vaccinations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. A suitable dose is an amount of a compound that, when administered as described above, is capable of promoting an anti-tumor immune response that is at least 10-50% above the basal (*i.e.,* untreated) level. Such response can be monitored by measuring the anti-tumor antibodies in a patient or by vaccine-dependent generation of cytolytic effector cells capable of killing the patient's tumor cells *in vitro*. Such vaccines should also be capable of causing an immune response that leads to an improved clinical outcome (*e.g.*, more frequent complete or partial remissions, or longer disease-free and/or overall survival) in vaccinated patients as compared to non-vaccinated patients. In general, for pharmaceutical compositions and vaccines comprising one or more polypeptides, the amount of each polypeptide present in a dose ranges from about 100 µg to 5 mg. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.1 mL to about 5 mL.

In general, an appropriate dosage and treatment regimen provides the active compound(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit. Such a response can be monitored by establishing an improved clinical outcome (*e.g.*, more frequent complete or partial remissions, or longer disease-free

and/or overall survival) in treated patients as compared to non-treated patients. Increases in preexisting immune responses to WT1 generally correlate with an improved clinical outcome. Such immune responses may generally be evaluated using standard proliferation, cytotoxicity or cytokine assays, which may be performed using samples obtained from a patient before and after treatment.

Within further aspects, methods for inhibiting the development of a malignant disease associated with WT1 expression involve the administration of autologous T cells that have been activated in response to a WT1 polypeptide or WT1-expressing APC, as described above. Such T cells may be $CD4^+$ and/or $CD8^+$, and may be proliferated as described above. The T cells may be administered to the individual in an amount effective to inhibit the development of a malignant disease. Typically, about 1×10^9 to 1×10^{11} T cells/M^2 are administered intravenously, intracavitary or in the bed of a resected tumor. It will be evident to those skilled in the art that the number of cells and the frequency of administration will be dependent upon the response of the patient.

Within certain embodiments, T cells may be stimulated prior to an autologous bone marrow transplantation. Such stimulation may take place *in vivo* or *in vitro*. For *in vitro* stimulation, bone marrow and/or peripheral blood (or a fraction of bone marrow or peripheral blood) obtained from a patient may be contacted with a WT1 polypeptide, a polynucleotide encoding a WT1 polypeptide and/or an APC that expresses a WT1 polypeptide under conditions and for a time sufficient to permit the stimulation of T cells as described above. Bone marrow, peripheral blood stem cells and/or WT1-specific T cells may then be administered to a patient using standard techniques.

Within related embodiments, T cells of a related or unrelated donor may be stimulated prior to a syngeneic or allogeneic (related or unrelated) bone marrow transplantation. Such stimulation may take place *in vivo* or *in vitro*. For *in vitro* stimulation, bone marrow and/or peripheral blood (or a fraction of bone marrow or peripheral blood) obtained from a related or unrelated donor may be contacted with a WT1 polypeptide, WT1 polynucleotide and/or APC that expresses a WT1 polypeptide under conditions and for a time sufficient to permit the stimulation of T cells as

described above. Bone marrow, peripheral blood stem cells and/or WT1-specific T cells may then be administered to a patient using standard techniques.

Within other embodiments, WT1-specific T cells as described herein may be used to remove cells expressing WT1 from autologous bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood (*e.g.*, $CD34^+$ enriched peripheral blood (PB) prior to administration to a patient). Such methods may be performed by contacting bone marrow or PB with such T cells under conditions and for a time sufficient to permit the reduction of WT1 expressing cells to less than 10%, preferably less than 5% and more preferably less than 1%, of the total number of myeloid or lymphatic cells in the bone marrow or peripheral blood. The extent to which such cells have been removed may be readily determined by standard methods such as, for example, qualitative and quantitative PCR analysis, morphology, immunohistochemistry and FACS analysis. Bone marrow or PB (or a fraction thereof) may then be administered to a patient using standard techniques.

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DIAGNOSTIC METHODS

The present invention further provides methods for detecting a malignant disease associated with WT1 expression, and for monitoring the effectiveness of an immunization or therapy for such a disease. Such methods are based on the discovery, within the present invention, that an immune response specific for WT1 protein can be detected in patients afflicted with such diseases, and that methods which enhance such immune responses may provide a preventive or therapeutic benefit.

To determine the presence or absence of a malignant disease associated with WT1 expression, a patient may be tested for the level of T cells specific for WT1. Within certain methods, a biological sample comprising $CD4^+$ and/or $CD8^+$ T cells isolated from a patient is incubated with a WT1 polypeptide, a polynucleotide encoding a WT1 polypeptide and/or an APC that expresses a WT1 polypeptide, and the presence or absence of specific activation of the T cells is detected, as described herein. Suitable biological samples include, but are not limited to, isolated T cells. For example, T cells may be isolated from a patient by routine techniques (such as by Ficoll/Hypaque density

gradient centrifugation of peripheral blood lymphocytes). T cells may be incubated *in vitro* for 2-9 days (typically 4 days) at 37°C with WT1 polypeptide (*e.g.*, 5 - 25 μg/ml). It may be desirable to incubate another aliquot of a T cell sample in the absence of WT1 polypeptide to serve as a control. For $CD4^+$ T cells, activation is preferably detected by evaluating proliferation of the T cells. For $CD8^+$ T cells, activation is preferably detected by evaluating cytolytic activity. A level of proliferation that is at least two fold greater and/or a level of cytolytic activity that is at least 20% greater than in disease-free patients indicates the presence of a malignant disease associated with WT1 expression. Further correlation may be made, using methods well known in the art, between the level of proliferation and/or cytolytic activity and the predicted response to therapy. In particular, patients that display a higher antibody, proliferative and/or lytic response may be expected to show a greater response to therapy.

Within other methods, a biological sample obtained from a patient is tested for the level of antibody specific for WT1. The biological sample is incubated with a WT1 polypeptide, a polynucleotide encoding a WT1 polypeptide and/or an APC that expresses a WT1 polypeptide under conditions and for a time sufficient to allow immunocomplexes to form. Immunocomplexes formed between the WT1 polypeptide and antibodies in the biological sample that specifically bind to the WT1 polypeptide are then detected. A biological sample for use within such methods may be any sample obtained from a patient that would be expected to contain antibodies. Suitable biological samples include blood, sera, ascites, bone marrow, pleural effusion, and cerebrospinal fluid.

The biological sample is incubated with the WT1 polypeptide in a reaction mixture under conditions and for a time sufficient to permit immunocomplexes to form between the polypeptide and antibodies specific for WT1. For example, a biological sample and WT1 polypeptide may be incubated at 4°C for 24-48 hours.

Following the incubation, the reaction mixture is tested for the presence of immunocomplexes. Detection of immunocomplexes formed between the WT1 polypeptide and antibodies present in the biological sample may be accomplished by a variety of known techniques, such as radioimmunoassays (RIA) and enzyme linked

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immunosorbent assays (ELISA). Suitable assays are well known in the art and are amply described in the scientific and patent literature (*e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988). Assays that may be used include, but are not limited to, the double monoclonal antibody sandwich immunoassay technique of David et al. (U.S. Patent 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide et al., in Kirkham and Hunter, eds., *Radioimmunoassay Methods*, E. and S. Livingstone, Edinburgh, 1970); the "western blot" method of Gordon et al. (U.S. Patent 4,452,901); immunoprecipitation of labeled ligand (Brown et al., *J. Biol. Chem. 255*:4980-4983, 1980); enzyme-linked immunosorbent assays as described by, for example, Raines and Ross (*J. Biol. Chem. 257*:5154-5160, 1982); immunocytochemical techniques, including the use of fluorochromes (Brooks et al., *Clin. Exp. Immunol. 39*: 477, 1980); and neutralization of activity (Bowen-Pope et al., *Proc. Natl. Acad. Sci. USA 81*:2396-2400, 1984). Other immunoassays include, but are not limited to, those described in U.S. Patent Nos.: 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876.

For detection purposes, WT1 polypeptide may either be labeled or unlabeled. Unlabeled WT1 polypeptide may be used in agglutination assays or in combination with labeled detection reagents that bind to the immunocomplexes (*e.g.*, anti-immunoglobulin, protein G, protein A or a lectin and secondary antibodies, or antigen-binding fragments thereof, capable of binding to the antibodies that specifically bind to the WT1 polypeptide). If the WT1 polypeptide is labeled, the reporter group may be any suitable reporter group known in the art, including radioisotopes, fluorescent groups, luminescent groups, enzymes, biotin and dye particles.

Within certain assays, unlabeled WT1 polypeptide is immobilized on a solid support. The solid support may be any material known to those of ordinary skill in the art to which the polypeptide may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a

magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Patent No. 5,359,681. The polypeptide may be immobilized on the solid support using a variety of techniques known to those of skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the antigen and functional groups on the support or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the WT1 polypeptide, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1 hour and about 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of polypeptide ranging from about 10 ng to about 10 μg, and preferably about 100 ng to about 1 μg, is sufficient to immobilize an adequate amount of polypeptide.

Following immobilization, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin, Tween 20™ (Sigma Chemical Co., St. Louis, MO), heat-inactivated normal goat serum (NGS), or BLOTTO (buffered solution of nonfat dry milk which also contains a preservative, salts, and an antifoaming agent). The support is then incubated with a biological sample suspected of containing specific antibody. The sample can be applied neat, or, more often, it can be diluted, usually in a buffered solution which contains a small amount (0.1%-5.0% by weight) of protein, such as BSA, NGS, or BLOTTO. In general, an appropriate contact time (*i.e.*, incubation time) is a period of time that is sufficient to detect the presence of antibody that specifically binds WT1 within a sample containing such an antibody. Preferably, the contact time is sufficient to achieve a level of binding that is at least about 95% of that achieved at equilibrium between bound and unbound antibody. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be

readily determined by assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20™. A detection reagent that binds to the immunocomplexes and that comprises a reporter group may then be added. The detection reagent is incubated with the immunocomplex for an amount of time sufficient to detect the bound antibody. An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period of time. Unbound detection reagent is then removed and bound detection reagent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups (*e.g.,* horseradish peroxidase, beta-galactosidase, alkaline phosphatase and glucose oxidase) may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products. Regardless of the specific method employed, a level of bound detection reagent that is at least two fold greater than background (*i.e.,* the level observed for a biological sample obtained from a disease-free individual) indicates the presence of a malignant disease associated with WT1 expression.

In general, methods for monitoring the effectiveness of an immunization or therapy involve monitoring changes in the level of antibodies or T cells specific for WT1 in the patient. Methods in which antibody levels are monitored may comprise the steps of: (a) incubating a first biological sample, obtained from a patient prior to a therapy or immunization, with a WT1 polypeptide, wherein the incubation is performed under conditions and for a time sufficient to allow immunocomplexes to form; (b) detecting immunocomplexes formed between the WT1 polypeptide and antibodies in the biological sample that specifically bind to the WT1 polypeptide; (c) repeating steps

(a) and (b) using a second biological sample taken from the patient following therapy or immunization; and (d) comparing the number of immunocomplexes detected in the first and second biological samples. Alternatively, a polynucleotide encoding a WT1 polypeptide, or an APC expressing a WT1 polypeptide may be employed in place of the WT1 polypeptide. Within such methods, immunocomplexes between the WT1 polypeptide encoded by the polynucleotide, or expressed by the APC, and antibodies in the biological sample are detected.

Methods in which T cell activation and/or the number of WT1 specific precursors are monitored may comprise the steps of: (a) incubating a first biological sample comprising CD4+ and/or CD8+ cells (*e.g.,* bone marrow, peripheral blood or a fraction thereof), obtained from a patient prior to a therapy or immunization, with a WT1 polypeptide, wherein the incubation is performed under conditions and for a time sufficient to allow specific activation, proliferation and/or lysis of T cells; (b) detecting an amount of activation, proliferation and/or lysis of the T cells; (c) repeating steps (a) and (b) using a second biological sample comprising CD4+ and/or CD8+ T cells, and taken from the same patient following therapy or immunization; and (d) comparing the amount of activation, proliferation and/or lysis of T cells in the first and second biological samples. Alternatively, a polynucleotide encoding a WT1 polypeptide, or an APC expressing a WT1 polypeptide may be employed in place of the WT1 polypeptide.

A biological sample for use within such methods may be any sample obtained from a patient that would be expected to contain antibodies, CD4+ T cells and/or CD8+ T cells. Suitable biological samples include blood, sera, ascites, bone marrow, pleural effusion and cerebrospinal fluid. A first biological sample may be obtained prior to initiation of therapy or immunization or part way through a therapy or vaccination regime. The second biological sample should be obtained in a similar manner, but at a time following additional therapy or immunization. The second biological sample may be obtained at the completion of, or part way through, therapy or immunization, provided that at least a portion of therapy or immunization takes place between the isolation of the first and second biological samples.

Incubation and detection steps for both samples may generally be performed as described above. A statistically significant increase in the number of immunocomplexes in the second sample relative to the first sample reflects successful therapy or immunization.

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

Identification of an Immune Response to WT1 in Patients with Hematological Malignancies

This Example illustrates the identification of an existent immune response in patients with a hematological malignancy.

To evaluate the presence of preexisting WT1 specific antibody responses in patients, sera of patients with AML, ALL, CML and severe aplastic anemia were analyzed using Western blot analysis. Sera were tested for the ability to immunoprecipitate WT1 from the human leukemic cell line K562 (American Type Culture Collection, Manassas, VA). In each case, immunoprecipitates were separated by gel electrophoresis, transferred to membrane and probed with the anti WT-1 antibody WT180 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). This Western blot analysis identified potential WT1 specific antibodies in patients with hematological malignancy. A representative Western blot showing the results for a patient with AML is shown in Figure 2. A 52 kD protein in the immunoprecipitate generated using the patient sera was recognized by the WT1 specific antibody. The 52 kD protein migrated at the same size as the positive control.

Example 2

Induction of Antibodies to WT1 in Mice Immunized with Cell Lines Expressing WT1

This Example illustrates the use of cells expressing WT1 to induce a WT1 specific antibody response *in vivo*.

Detection of existent antibodies to WT1 in patients with leukemia strongly implied that it is possible to immunize to WT1 protein to elicit immunity to WT1. To test whether immunity to WT1 can be generated by vaccination, mice were injected with TrampC, a WT1 positive tumor cell line of B6 origin. Briefly, male B6 mice were immunized with 5 x 10^6 TrampC cells subcutaneously and boosted twice

with 5 x 10^6 cells at three week intervals. Following immunization to TrampC, a WT1 specific antibody response in the immunized animals was detectable. A representative Western blot is shown in Figure 3. These results show that immunization to WT1 protein can elicit an immune response to WT1 protein.

Example 3

Induction of Th and Antibody Responses in Mice Immunized with WT1 Peptides

This Example illustrates the ability of immunization with WT1 peptides to elicit an immune response specific for WT1.

Peptides suitable for eliciting Ab and proliferative T cell responses were identified according to the Tsites program (Rothbard and Taylor, *EMBO J.* 7:93-100, 1988; Deavin et al., *Mol. Immunol.* 33:145-155, 1996), which searches for peptide motifs that have the potential to elicit Th responses. Peptides shown in Table I were synthesized and sequenced.

Table I
WT1 Peptides

Peptide	Sequence	Comments
Mouse: p6-22	RDLNALLPAVSSLGGGG (SEQ ID NO:13)	1 mismatch relative to human WT1 sequence
Human: p6-22	RDLNALLPAVPSLGGGG (SEQ ID NO:1)	
Human/mouse: p117-139	PSQASSGQARMFPNAPYLPSCLE (SEQ ID NOs: 2 and 3)	
Mouse: p244-262	GATLKGMAAGSSSSVKWTE (SEQ ID NO:14)	1 mismatch relative to human WT1 sequence
Human: p244-262	GATLKGVAAGSSSSVKWTE (SEQ ID NO:4)	
Human/mouse: p287-301	RIHTHGVFRGIQDVR (SEQ ID NOs: 15 and 16)	
Mouse: p299-313	VRRVSGVAPTLVRS (SEQ ID NO:17)	1 mismatch relative to human WT1 sequence
Human/mouse: p421-435	CQKKFARSDELVRHH (SEQ ID NOs: 19 and 20)	

For immunization, peptides were grouped as follows:

Group A: p6-22 human: 10.9mg in 1ml (10μl = 100μg)
p117-139 human/mouse: 7.6mg in 1ml (14μl = 100μg)
p244-262 human: 4.6.mg in 1ml (22μl = 100μg)

Group B: p287-301 human/mouse: 7.2mg in 1ml (14μl = 100μg)
mouse p299-313: 6.6.mg in 1ml (15μl = 100μg)
p421-435 human/mouse: 3.3mg in 1ml (30μl = 100μg)

Control: (FBL peptide 100μg) + CFA/IFA

Control: (CD45 peptide 100μg) + CFA/IFA

B6 mice were immunized with a group of WT1 peptides or with a control peptide. Peptides were dissolved in 1ml sterile water for injection, and B6 mice were immunized 3 times at time intervals of three weeks. Adjuvants used were CFA/IFA, GM-CSF, and Montinide. The presence of antibodies specific for WT1 was then determined as described in Examples 1 and 2, and proliferative T cell responses were evaluated using a standard thymidine incorporation assay, in which cells were cultured in the presence of antigen and proliferation was evaluated by measuring incorporated radioactivity (Chen et al., *Cancer Res. 54*:1065-1070, 1994).

Immunization of mice with the group of peptides designated as Group A elicited an antibody response to WT1 (Figure 4), and P117-139 elicited proliferative T cell responses (Figures 5A-5C). The stimulation indices (SI) varied between 8 and 72. Other peptides (P6-22 and P299-313) also were shown to elicit proliferative T cell responses. Immunization with P6-22 resulted in a stimulation index (SI) of 2.3 and immunization with P299-313 resulted in a SI of 3.3. Positive controls included ConA stimulated T cells, as well as T cells stimulated with known antigens, such as CD45 and FBL, and allogeneic T cell lines (DeBruijn et al., *Eur. J. Immunol. 21*:2963-2970, 1991).

These results show that vaccination with WT1 peptides can elicit antibody responses to WT1 protein and proliferative T cell responses to the immunizing peptides.

Example 4

Induction of CTL Responses in Mice Immunized with WT1 Peptides

This Example illustrates the ability of WT1 peptides to elicit CTL immunity.

Peptides (9-mers) with motifs appropriate for binding to class I MHC were identified using a BIMAS HLA peptide binding prediction analysis (Parker et al., *J. Immunol. 152*:163, 1994). Peptides identified within such analyses are shown in Tables II - XLIV. In each of these tables, the score reflects the theoretical binding affinity (half-time of dissociation) of the peptide to the MHC molecule indicated.

Peptides identified using the Tsites program (Rothbard and Taylor, *EMBO J. 7*:93-100, 1988; Deavin et al., *Mol. Immunol. 33*:145-155, 1996), which searches for peptide motifs that have the potential to elicit Th responses are further shown in Figures 8A and 8B, and Table XLV.

Table II
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA A1

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	137	CLESQPAIR (SEQ ID NO:47)	18.000
2	80	GAEPHEEQC (SEQ ID NO:87)	9.000
3	40	FAPPGASAY (SEQ ID NO:74)	5.000
4	354	QCDFKDCER (SEQ ID NO:162)	5.000
5	2	GSDVRDLNA (SEQ ID NO:101)	3.750

6	152	VTFDGTPSY (SEQ ID NO:244)	2.500
7	260	WTEGQSNHS (SEQ ID NO:247)	2.250
8	409	TSEKPFSCR (SEQ ID NO:232)	1.350
9	73	KQEPSWGGA (SEQ ID NO:125)	1.350
10	386	KTCQRKFSR (SEQ ID NO:128)	1.250
11	37	VLDFAPPGA (SEQ ID NO:241)	1.000
12	325	CAYPGCNKR (SEQ ID NO:44)	1.000
13	232	QLECMTWNQ (SEQ ID NO:167)	0.900
14	272	ESDNHTTPI (SEQ ID NO:71)	0.750
15	366	RSDQLKRHQ (SEQ ID NO:193)	0.750
16	222	SSDNLYQMT (SEQ ID NO:217)	0.750
17	427	RSDELVRHH (SEQ ID NO:191)	0.750
18	394	RSDHLKTHT (SEQ ID NO:192)	0.750
19	317	TSEKRPFMC (SEQ ID NO:233)	0.675
20	213	QALLLRTPY (SEQ ID NO:160)	0.500

Table III

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA A 0201

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	126	RMFPNAPYL (SEQ ID NO:185)	313.968
2	187	SLGEQQYSV (SEQ ID NO:214)	285.163
3	10	ALLPAVPSL (SEQ ID NO:34)	181.794

4	242	NLGATLKGV (SEQ ID NO:146)	159.970
5	225	NLYQMTSQL (SEQ ID NO:147)	68.360
6	292	GVFRGIQDV (SEQ ID NO:103)	51.790
7	191	QQYSVPPPV (SEQ ID NO:171)	22.566
8	280	ILCGAQYRI (SEQ ID NO:116)	17.736
9	235	CMTWNQMNL (SEQ ID NO:49)	15.428
10	441	NMTKLQLAL (SEQ ID NO:149)	15.428
11	7	DLNALLPAV (SEQ ID NO:58)	11.998
12	227	YQMTSQLEC (SEQ ID NO:251)	8.573
13	239	NQMNLGATL (SEQ ID NO:151)	8.014
14	309	TLVRSASET (SEQ ID NO:226)	7.452
15	408	KTSEKPFSC (SEQ ID NO:129)	5.743
16	340	LQMHSRKHT (SEQ ID NO:139)	4.752
17	228	QMTSQLECM (SEQ ID NO:169)	4.044
18	93	TVHFSGQFT (SEQ ID NO:235)	3.586
19	37	VLDFAPPGA (SEQ ID NO:241)	3.378
20	86	EQCLSAFTV (SEQ ID NO:69)	3.068

Table IV

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA A 0205

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	10	ALLPAVPSL (SEQ ID NO:34)	42.000

2	292	GVFRGIQDV (SEQ ID NO:103)	24.000
3	126	RMFPNAPYL (SEQ ID NO:185)	21.000
4	225	NLYQMTSQL (SEQ ID NO:147)	21.000
5	239	NQMNLGATL (SEQ ID NO:151)	16.800
6	302	RVPGVAPTL (SEQ ID NO:195)	14.000
7	441	NMTKLQLAL (SEQ ID NO:149)	7.000
8	235	CMTWNQMNL (SEQ ID NO:49)	7.000
9	187	SLGEQQYSV (SEQ ID NO:214)	6.000
10	191	QQYSVPPPV (SEQ ID NO:171)	4.800
11	340	LQMHSRKHT (SEQ ID NO:139)	4.080
12	242	NLGATLKGV (SEQ ID NO:146)	4.000
13	227	YQMTSQLEC (SEQ ID NO:251)	3.600
14	194	SVPPPVYGC (SEQ ID NO:218)	2.000
15	93	TVHFSGQFT (SEQ ID NO:235)	2.000
16	280	ILCGAQYRI (SEQ ID NO:116)	1.700
17	98	GQFTGTAGA (SEQ ID NO:99)	1.200
18	309	TLVRSASET (SEQ ID NO:226)	1.000
19	81	AEPHEEQCL (SEQ ID NO:30)	0.980
20	73	KQEPSWGGA (SEQ ID NO:125)	0.960

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Table V
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA A24

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	302	RVPGVAPTL (SEQ ID NO:195)	16.800
2	218	RTPYSSDNL (SEQ ID NO:194)	12.000
3	356	DFKDCERRF (SEQ ID NO:55)	12.000
4	126	RMFPNAPYL (SEQ ID NO:185)	9.600
5	326	AYPGCNKRY (SEQ ID NO:42)	7.500
6	270	GYESDNHT (SEQ ID NO:106)T	7.500
7	239	NQMNLGATL (SEQ ID NO:151)	7.200
8	10	ALLPAVPSL (SEQ ID NO:34)	7.200
9	130	NAPYLPSCL (SEQ ID NO:144)	7.200
10	329	GCNKRYFKL (SEQ ID NO:90)	6.600
11	417	RWPSCQKKF (SEQ ID NO:196)	6.600
12	47	AYGSLGGPA (SEQ ID NO:41)	6.000
13	180	DPMGQQGSL (SEQ ID NO:59)	6.000
14	4	DVRDLNALL (SEQ ID NO:62)	5.760
15	285	QYRIHTHGV (SEQ ID NO:175)	5.000
16	192	QYSVPPPVY (SEQ ID NO:176)	5.000
17	207	DSCTGSQAL (SEQ ID NO:61)	4.800
18	441	NMTKLQLAL (SEQ ID NO:149)	4.800
19	225	NLYQMTSQL (SEQ	4.000

		ID NO:147)	
20	235	CMTWNQMNL (SEQ ID NO:49)	4.000

Table VI
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA A3

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	436	NMHQRNMTK (SEQ ID NO:148)	40.000
2	240	QMNLGATLK (SEQ ID NO:168)	20.000
3	88	CLSAFTVHF (SEQ ID NO:48)	6.000
4	126	RMFPNAPYL (SEQ ID NO:185)	4.500
5	169	AQFPNHSFK (SEQ ID NO:36)	4.500
6	10	ALLPAVPSL (SEQ ID NO:34)	4.050
7	137	CLESQPAIR (SEQ ID NO:47)	4.000
8	225	NLYQMTSQL (SEQ ID NO:147)	3.000
9	32	AQWAPVLDF (SEQ ID NO:37)	2.700
10	280	ILCGAQYRI (SEQ ID NO:116)	2.700
11	386	KTCQRKFSR (SEQ ID NO:128)	1.800
12	235	CMTWNQMNL (SEQ ID NO:49)	1.200
13	441	NMTKLQLAL (SEQ ID NO:149)	1.200
14	152	VTFDGTPSY (SEQ ID NO:244)	1.000
15	187	SLGEQQYSV (SEQ ID NO:214)	0.900
16	383	FQCKTCQRK (SEQ ID NO:80)	0.600
17	292	GVFRGIQDV (SEQ	0.450

		ID NO:103)	
18	194	SVPPPVYGC (SEQ ID NO:218)	0.405
19	287	RIHTHGVFR (SEQ ID NO:182)	0.400
20	263	GQSNHSTGY (SEQ ID NO:100)	0.360

Table VII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA A68.1

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	100	FTGTAGACR (SEQ ID NO:84)	100.000
2	386	KTCQRKFSR (SEQ ID NO:128)	50.000
3	368	DQLKRHQRR (SEQ ID NO:60)	30.000
4	312	RSASETSEK (SEQ ID NO:190)	18.000
5	337	LSHLQMHSR (SEQ ID NO:141)	15.000
6	364	FSRSDQLKR (SEQ ID NO:83)	15.000
7	409	TSEKPFSCR (SEQ ID NO:232)	15.000
8	299	DVRRVPGVA (SEQ ID NO:63)	12.000
9	4	DVRDLNALL (SEQ ID NO:62)	12.000
10	118	SQASSGQAR (SEQ ID NO:216)	10.000
11	343	HSRKHTGEK (SEQ ID NO:111)	9.000
12	169	AQFPNHSFK (SEQ ID NO:36)	9.000
13	292	GVFRGIQDV (SEQ ID NO:103)	8.000
14	325	CAYPGCNKR (SEQ ID NO:44)	7.500
15	425	FARSDELVR (SEQ	7.500

		ID NO:75)	
16	354	QCDFKDCER (SEQ ID NO:162)	7.500
17	324	MCAYPGCNK (SEQ ID NO:142)	6.000
18	251	AAGSSSSVK (SEQ ID NO:28)	6.000
19	379	GVKPFQCKT (SEQ ID NO:104)	6.000
20	137	CLESQPAIR (SEQ ID NO:47)	5.000

Table VIII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA A 1101

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	386	KTCQRKFSR (SEQ ID NO:128)	1.800
2	169	AQFPNHSFK (SEQ ID NO:36)	1.200
3	436	NMHQRNMTK (SEQ ID NO:148)	0.800
4	391	KFSRSDHLK (SEQ ID NO:120)	0.600
5	373	HQRRHTGVK (SEQ ID NO:109)	0.600
6	383	FQCKTCQRK (SEQ ID NO:80)	0.600
7	363	RFSRSDQLK (SEQ ID NO:178)	0.600
8	240	QMNLGATLK (SEQ ID NO:168)	0.400
9	287	RIHTHGVFR (SEQ ID NO:182)	0.240
10	100	FTGTAGACR (SEQ ID NO:84)	0.200
11	324	MCAYPGCNK (SEQ ID NO:142)	0.200
12	251	AAGSSSSVK (SEQ ID NO:28)	0.200
13	415	SCRWPSCQK (SEQ	0.200

		ID NO:201)	
14	118	SQASSGQAR (SEQ ID NO:216)	0.120
15	292	GVFRGIQDV (SEQ ID NO:103)	0.120
16	137	CLESQPAIR (SEQ ID NO:47)	0.080
17	425	FARSDELVR (SEQ ID NO:75)	0.080
18	325	CAYPGCNKR (SEQ ID NO:44)	0.080
19	312	RSASETSEK (SEQ ID NO:190)	0.060
20	65	PPPPHSFI (SEQ ID NO:156)K	0.060

Table IX
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA A 3101

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	386	KTCQRKFSR (SEQ ID NO:128)	9.000
2	287	RIHTHGVFR (SEQ ID NO:182)	6.000
3	137	CLESQPAIR (SEQ ID NO:47)	2.000
4	118	SQASSGQAR (SEQ ID NO:216)	2.000
5	368	DQLKRHQRR (SEQ ID NO:60)	1.200
6	100	FTGTAGACR (SEQ ID NO:84)	1.000
7	293	VFRGIQDVR (SEQ ID NO:238)	0.600
8	325	CAYPGCNKR (SEQ ID NO:44)	0.600
9	169	AQFPNHSFK (SEQ ID NO:36)	0.600
10	279	PILCGAQYR (SEQ ID NO:155)	0.400
11	436	NMHQRNMTK (SEQ	0.400

		ID NO:148)	
12	425	FARSDELVR (SEQ ID NO:75)	0.400
13	32	AQWAPVLDF (SEQ ID NO:37)	0.240
14	240	QMNLGATLK (SEQ ID NO:168)	0.200
15	354	QCDFKDCER (SEQ ID NO:162)	0.200
16	373	HQRRHTGVK (SEQ ID NO:109)	0.200
17	383	FQCKTCQRK (SEQ ID NO:80)	0.200
18	313	SASETSEKR (SEQ ID NO:197)	0.200
19	358	KDCERRFSR (SEQ ID NO:118)	0.180
20	391	KFSRSDHLK (SEQ ID NO:120)	0.180

Table X
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA A 3302

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	337	LSHLQMHSR (SEQ ID NO:141)	15.000
2	409	TSEKPFSCR (SEQ ID NO:232)	15.000
3	364	FSRSDQLKR (SEQ ID NO:83)	15.000
4	137	CLESQPAIR (SEQ ID NO:47)	9.000
5	368	DQLKRHQRR (SEQ ID NO:60)	9.000
6	287	RIHTHGVFR (SEQ ID NO:182)	4.500
7	210	TGSQALLLR (SEQ ID NO:223)	3.000
8	425	FARSDELVR (SEQ ID NO:75)	3.000
9	313	SASETSEKR (SEQ ID	3.000

		NO:197)	
10	293	VFRGIQDVR (SEQ ID NO:238)	3.000
11	354	QCDFKDCER (SEQ ID NO:162)	3.000
12	100	FTGTAGACR (SEQ ID NO:84)	3.000
13	118	SQASSGQAR (SEQ ID NO:216)	3.000
14	325	CAYPGCNKR (SEQ ID NO:44)	3.000
15	207	DSCTGSQAL (SEQ ID NO:61)	1.500
16	139	ESQPAIRNQ (SEQ ID NO:72)	1.500
17	299	DVRRVPGVA (SEQ ID NO:63)	1.500
18	419	PSCQKKFAR (SEQ ID NO:159)	1.500
19	272	ESDNHTTPI (SEQ ID NO:71)	1.500
20	4	DVRDLNALL (SEQ ID NO:62)	1.500

Table XI
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B14

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	362	RRFSRSDQL (SEQ ID NO:187)	1000.000
2	332	KRYFKLSHL (SEQ ID NO:127)	300.000
3	423	KKFARSDEL (SEQ ID NO:122)	150.000
4	390	RKFSRSDHL (SEQ ID NO:183)	150.000
5	439	QRNMTKLQL (SEQ ID NO:173)	20.000
6	329	GCNKRYFKL (SEQ ID NO:90)	10.000
7	10	ALLPAVPSL (SEQ ID	10.000

		NO:34)	
8	180	DPMGQQGSL (SEQ ID NO:59)	9.000
9	301	RRVPGVAPT (SEQ ID NO:189)	6.000
10	126	RMFPNAPYL (SEQ ID NO:185)	5.000
11	371	KRHQRRHTG (SEQ ID NO:126)	5.000
12	225	NLYQMTSQL (SEQ ID NO:147)	5.000
13	144	IRNQGYSTV (SEQ ID NO:117)	4.000
14	429	DELVRHHNM (SEQ ID NO:53)	3.000
15	437	MHQRNMTKL (SEQ ID NO:143)	3.000
16	125	ARMFPNAPY (SEQ ID NO:38)	3.000
17	239	NQMNLGATL (SEQ ID NO:151)	3.000
18	286	YRIHTHGVF (SEQ ID NO:252)	3.000
19	174	HSFKHEDPM (SEQ ID NO:110)	3.000
20	372	RHQRRHTGV (SEQ ID NO:181)	3.000

Table XII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B40

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	81	AEPHEEQCL (SEQ ID NO:30)	40.000
2	429	DELVRHHNM (SEQ ID NO:53)	24.000
3	410	SEKPFSCRW (SEQ ID NO:207)	20.000
4	318	SEKRPFMCA (SEQ ID NO:208)	15.000
5	233	LECMTWNQM (SEQ	12.000

		ID NO:131)	
6	3	SDVRDLNAL (SEQ ID NO:206)	10.000
7	349	GEKPYQCDF (SEQ ID NO:91)	8.000
8	6	RDLNALLPA (SEQ ID NO:177)	5.000
9	85	EEQCLSAFT (SEQ ID NO:65)	4.000
10	315	SETSEKRPF (SEQ ID NO:209)	4.000
11	261	TEGQSNHST (SEQ ID NO:221)	4.000
12	23	GCALPVSGA (SEQ ID NO:89)	3.000
13	38	LDFAPPGAS (SEQ ID NO:130)	3.000
14	273	SDNHTTPIL (SEQ ID NO:204)	2.500
15	206	TDSCTGSQA (SEQ ID NO:220)	2.500
16	24	CALPVSGAA (SEQ ID NO:43)	2.000
17	98	GQFTGTAGA (SEQ ID NO:99)	2.000
18	30	GAAQWAPVL (SEQ ID NO:86)	2.000
19	84	HEEQCLSAF (SEQ ID NO:107)	2.000
20	26	LPVSGAAQW (SEQ ID NO:138)	2.000

Table XIII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B60

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	81	AEPHEEQCL (SEQ ID NO:30)	160.000
2	3	SDVRDLNAL (SEQ ID NO:206)	40.000
3	429	DELVRHHNM (SEQ	40.000

		ID NO:53)	
4	233	LECMTWNQM (SEQ ID NO:131)	22.000
5	273	SDNHTTPIL (SEQ ID NO:204)	20.000
6	209	CTGSQALLL (SEQ ID NO:52)	8.000
7	30	GAAQWAPVL (SEQ ID NO:86)	8.000
8	318	SEKRPFMCA (SEQ ID NO:208)	8.000
9	180	DPMGQQGSL (SEQ ID NO:59)	8.000
10	138	LESQPAIRN (SEQ ID NO:132)	5.280
11	239	NQMNLGATL (SEQ ID NO:151)	4.400
12	329	GCNKRYFKL (SEQ ID NO:90)	4.400
13	130	NAPYLPSCL (SEQ ID NO:144)	4.400
14	85	EEQCLSAFT (SEQ ID NO:65)	4.400
15	208	SCTGSQALL (SEQ ID NO:202)	4.000
16	207	DSCTGSQAL (SEQ ID NO:61)	4.000
17	218	RTPYSSDNL (SEQ ID NO:194)	4.000
18	261	TEGQSNHST (SEQ ID NO:221)	4.000
19	18	LGGGGGCAL (SEQ ID NO:134)	4.000
20	221	YSSDNLYQM (SEQ ID NO:253)	2.200

Table XIV
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B61

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	318	SEKRPFMCA (SEQ	20.000

		ID NO:208)	
2	429	DELVRHHNM (SEQ ID NO:53)	16.000
3	298	QDVRRVPGV (SEQ ID NO:164)	10.000
4	81	AEPHEEQCL (SEQ ID NO:30)	8.000
5	233	LECMTWNQM (SEQ ID NO:131)	8.000
6	6	RDLNALLPA (SEQ ID NO:177)	5.500
7	85	EEQCLSAFT (SEQ ID NO:65)	4.000
8	261	TEGQSNHST (SEQ ID NO:221)	4.000
9	206	TDSCTGSQA (SEQ ID NO:220)	2.500
10	295	RGIQDVRRV (SEQ ID NO:179)	2.200
11	3	SDVRDLNAL (SEQ ID NO:206)	2.000
12	250	VAAGSSSSV (SEQ ID NO:236)	2.000
13	29	SGAAQWAPV (SEQ ID NO:211)	2.000
14	315	SETSEKRPF (SEQ ID NO:209)	1.600
15	138	LESQPAIRN (SEQ ID NO:132)	1.200
16	244	GATLKGVAA (SEQ ID NO:88)	1.100
17	20	GGGGCALPV (SEQ ID NO:92)	1.100
18	440	RNMTKLQLA (SEQ ID NO:186)	1.100
19	23	GCALPVSGA (SEQ ID NO:89)	1.100
20	191	QQYSVPPPV (SEQ ID NO:171)	1.000

Table XV
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B62

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	146	NQGYSTVTF (SEQ ID NO:150)	211.200
2	32	AQWAPVLDF (SEQ ID NO:37)	96.000
3	263	GQSNHSTGY (SEQ ID NO:100)	96.000
4	88	CLSAFTVHF (SEQ ID NO:48)	96.000
5	17	SLGGGGGCA (SEQ ID NO:215)	9.600
6	239	NQMNLGATL (SEQ ID NO:151)	8.800
7	191	QQYSVPPPV (SEQ ID NO:171)	8.000
8	98	GQFTGTAGA (SEQ ID NO:99)	8.000
9	384	QCKTCQRKF (SEQ ID NO:163)	6.000
10	40	FAPPGASAY (SEQ ID NO:74)	4.800
11	227	YQMTSQLEC (SEQ ID NO:251)	4.800
12	187	SLGEQQYSV (SEQ ID NO:214)	4.400
13	86	EQCLSAFTV (SEQ ID NO:69)	4.400
14	152	VTFDGTPSY (SEQ ID NO:244)	4.400
15	101	TGTAGACRY (SEQ ID NO:224)	4.000
16	242	NLGATLKGV (SEQ ID NO:146)	4.000
17	92	FTVHFSGQF (SEQ ID NO:85)	4.000
18	7	DLNALLPAV (SEQ ID NO:58)	4.000
19	123	GQARMFPNA (SEQ	4.000

		ID NO:98)	
20	280	ILCGAQYRI (SEQ ID NO:116)	3.120

Table XVI
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B7

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	180	DPMGQQGSL (SEQ ID NO:59)	240.000
2	4	DVRDLNALL (SEQ ID NO:62)	200.000
3	302	RVPGVAPTL (SEQ ID NO:195)	20.000
4	30	GAAQWAPVL (SEQ ID NO:86)	12.000
5	239	NQMNLGATL (SEQ ID NO:151)	12.000
6	130	NAPYLPSCL (SEQ ID NO:144)	12.000
7	10	ALLPAVPSL (SEQ ID NO:34)	12.000
8	299	DVRRVPGVA (SEQ ID NO:63)	5.000
9	208	SCTGSQALL (SEQ ID NO:202)	4.000
10	303	VPGVAPTLV (SEQ ID NO:242)	4.000
11	18	LGGGGGCAL (SEQ ID NO:134)	4.000
12	218	RTPYSSDNL (SEQ ID NO:194)	4.000
13	207	DSCTGSQAL (SEQ ID NO:61)	4.000
14	209	CTGSQALLL (SEQ ID NO:52)	4.000
15	329	GCNKRYFKL (SEQ ID NO:90)	4.000
16	235	CMTWNQMNL (SEQ ID NO:49)	4.000
17	441	NMTKLQLAL (SEQ	4.000

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		ID NO:149)	
18	126	RMFPNAPYL (SEQ ID NO:185)	4.000
19	225	NLYQMTSQL (SEQ ID NO:147)	4.000
20	143	AIRNQGYST (SEQ ID NO:33)	3.000

Table XVII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B8

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	329	GCNKRYFKL (SEQ ID NO:90)	16.000
2	4	DVRDLNALL (SEQ ID NO:62)	12.000
3	316	ETSEKRPFM (SEQ ID NO:73)	3.000
4	180	DPMGQQGSL (SEQ ID NO:59)	1.600
5	208	SCTGSQALL (SEQ ID NO:202)	0.800
6	130	NAPYLPSCL (SEQ ID NO:144)	0.800
7	244	GATLKGVAA (SEQ ID NO:88)	0.800
8	30	GAAQWAPVL (SEQ ID NO:86)	0.800
9	299	DVRRVPGVA (SEQ ID NO:63)	0.400
10	420	SCQKKFARS (SEQ ID NO:200)	0.400
11	387	TCQRKFSRS (SEQ ID NO:219)	0.400
12	225	NLYQMTSQL (SEQ ID NO:147)	0.400
13	141	QPAIRNQGY (SEQ ID NO:170)	0.400
14	10	ALLPAVPSL (SEQ ID NO:34)	0.400
15	207	DSCTGSQAL (SEQ	0.400

		ID NO:61)	
16	384	QCKTCQRKF (SEQ ID NO:163)	0.400
17	136	SCLESQPAI (SEQ ID NO:198)	0.300
18	347	HTGEKPYQC (SEQ ID NO:112)	0.300
19	401	HTRTHTGKT (SEQ ID NO:114)	0.200
20	332	KRYFKLSHL (SEQ ID NO:127)	0.200

Table XVIII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 2702

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	332	KRYFKLSHL (SEQ ID NO:127)	900.000
2	362	RRFSRSDQL (SEQ ID NO:187)	900.000
3	286	YRIHTHGVF (SEQ ID NO:252)	200.000
4	125	ARMFPNAPY (SEQ ID NO:38)	200.000
5	375	RRHTGVKPF (SEQ ID NO:188)	180.000
6	32	AQWAPVLDF (SEQ ID NO:37)	100.000
7	301	RRVPGVAPT (SEQ ID NO:189)	60.000
8	439	QRNMTKLQL (SEQ ID NO:173)	60.000
9	126	RMFPNAPYL (SEQ ID NO:185)	22.500
10	426	ARSDELVRH (SEQ ID NO:39)	20.000
11	146	NQGYSTVTF (SEQ ID NO:150)	20.000
12	144	IRNQGYSTV (SEQ ID NO:117)	20.000
13	389	QRKFSRSDH (SEQ	20.000

		ID NO:172)	
14	263	GQSNHSTGY (SEQ ID NO:100)	20.000
15	416	CRWPSCQKK (SEQ ID NO:50)	20.000
16	191	QQYSVPPPV (SEQ ID NO:171)	10.000
17	217	LRTPYSSDN (SEQ ID NO:140)	10.000
18	107	CRYGPFGPP (SEQ ID NO:51)	10.000
19	98	GQFTGTAGA (SEQ ID NO:99)	10.000
20	239	NQMNLGATL (SEQ ID NO:151)	6.000

Table XIX
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 2705

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	332	KRYFKLSHL (SEQ ID NO:127)	30000.000
2	362	RRFSRSDQL (SEQ ID NO:187)	30000.000
3	416	CRWPSCQKK (SEQ ID NO:50)	10000.000
4	439	QRNMTKLQL (SEQ ID NO:173)	2000.000
5	286	YRIHTHGVF (SEQ ID NO:252)	1000.000
6	125	ARMFPNAPY (SEQ ID NO:38)	1000.000
7	294	FRGIQDVRR (SEQ ID NO:81)	1000.000
8	432	VRHHNMHQR (SEQ ID NO:243)	1000.000
9	169	AQFPNHSFK (SEQ ID NO:36)	1000.000
10	375	RRHTGVKPF (SEQ ID NO:188)	900.000
11	126	RMFPNAPYL (SEQ	750.000

		ID NO:185)	
12	144	IRNQGYSTV (SEQ ID NO:117)	600.000
13	301	RRVPGVAPT (SEQ ID NO:189)	600.000
14	32	AQWAPVLDF (SEQ ID NO:37)	500.000
15	191	QQYSVPPPV (SEQ ID NO:171)	300.000
16	373	HQRRHTGVK (SEQ ID NO:109)	200.000
17	426	ARSDELVRH (SEQ ID NO:39)	200.000
18	383	FQCKTCQRK (SEQ ID NO:80)	200.000
19	239	NQMNLGATL (SEQ ID NO:151)	200.000
20	389	QRKFSRSDH (SEQ ID NO:172)	200.000

Table XX
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 3501

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	278	TPILCGAQY (SEQ ID NO:227)	40.000
2	141	QPAIRNQGY (SEQ ID NO:170)	40.000
3	219	TPYSSDNLY (SEQ ID NO:231)	40.000
4	327	YPGCNKRYF (SEQ ID NO:250)	20.000
5	163	TPSHHAAQF (SEQ ID NO:228)	20.000
6	180	DPMGQQGSL (SEQ ID NO:59)	20.000
7	221	YSSDNLYQM (SEQ ID NO:253)	20.000
8	26	LPVSGAAQW (SEQ ID NO:138)	10.000
9	174	HSFKHEDPM (SEQ	10.000

		ID NO:110)	
10	82	EPHEEQCLS (SEQ ID NO:68)	6.000
11	213	QALLLRTPY (SEQ ID NO:160)	6.000
12	119	QASSGQARM (SEQ ID NO:161)	6.000
13	4	DVRDLNALL (SEQ ID NO:62)	6.000
14	40	FAPPGASAY (SEQ ID NO:74)	6.000
15	120	ASSGQARMF (SEQ ID NO:40)	5.000
16	207	DSCTGSQAL (SEQ ID NO:61)	5.000
17	303	VPGVAPTLV (SEQ ID NO:242)	4.000
18	316	ETSEKRPFM (SEQ ID NO:73)	4.000
19	152	VTFDGTPSY (SEQ ID NO:244)	4.000
20	412	KPFSCRWPS (SEQ ID NO:123)	4.000

Table XXI
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 3701

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	3	SDVRDLNAL (SEQ ID NO:206)	40.000
2	273	SDNHTTPIL (SEQ ID NO:204)	40.000
3	81	AEPHEEQCL (SEQ ID NO:30)	10.000
4	298	QDVRRVPGV (SEQ ID NO:164)	8.000
5	428	SDELVRHHN (SEQ ID NO:203)	6.000
6	85	EEQCLSAFT (SEQ ID NO:65)	5.000
7	208	SCTGSQALL (SEQ ID	5.000

		NO:202)	
8	4	DVRDLNALL (SEQ ID NO:62)	5.000
9	209	CTGSQALLL (SEQ ID NO:52)	5.000
10	38	LDFAPPGAS (SEQ ID NO:130)	4.000
11	223	SDNLYQMTS (SEQ ID NO:205)	4.000
12	179	EDPMGQQGS (SEQ ID NO:64)	4.000
13	206	TDSCTGSQA (SEQ ID NO:220)	4.000
14	6	RDLNALLPA (SEQ ID NO:177)	4.000
15	84	HEEQCLSAF (SEQ ID NO:107)	2.000
16	233	LECMTWNQM (SEQ ID NO:131)	2.000
17	429	DELVRHHNM (SEQ ID NO:53)	2.000
18	315	SETSEKRPF (SEQ ID NO:209)	2.000
19	349	GEKPYQCDF (SEQ ID NO:91)	2.000
20	302	RVPGVAPTL (SEQ ID NO:195)	1.500

Table XXII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 3801

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	437	MHQRNMTKL (SEQ ID NO:143)	36.000
2	434	HHNMHQRNM (SEQ ID NO:108)	6.000
3	372	RHQRRHTGV (SEQ ID NO:181)	6.000
4	180	DPMGQQGSL (SEQ ID NO:59)	4.000
5	433	RHHNMHQRN (SEQ	3.900

		ID NO:180)	
6	165	SHHAAQFPN (SEQ ID NO:213)	3.900
7	202	CHTPTDSCT (SEQ ID NO:45)	3.000
8	396	DHLKTHTRT (SEQ ID NO:57)	3.000
9	161	GHTPSHHAA (SEQ ID NO:94)	3.000
10	302	RVPGVAPTL (SEQ ID NO:195)	2.600
11	417	RWPSCQKKF (SEQ ID NO:196)	2.400
12	327	YPGCNKRYF (SEQ ID NO:250)	2.400
13	208	SCTGSQALL (SEQ ID NO:202)	2.000
14	163	TPSHHAAQF (SEQ ID NO:228)	2.000
15	120	ASSGQARMF (SEQ ID NO:40)	2.000
16	18	LGGGGGCAL (SEQ ID NO:134)	2.000
17	177	KHEDPMGQQ (SEQ ID NO:121)	1.800
18	83	PHEEQCLSA (SEQ ID NO:154)	1.800
19	10	ALLPAVPSL (SEQ ID NO:34)	1.300
20	225	NLYQMTSQL (SEQ ID NO:147)	1.300

Table XXIII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 3901

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	437	MHQRNMTKL (SEQ ID NO:143)	135.000
2	332	KRYFKLSHL (SEQ ID NO:127)	45.000
3	434	HHNMHQRNM (SEQ	30.000

		ID NO:108)	
4	362	RRFSRSDQL (SEQ ID NO:187)	30.000
5	372	RHQRRHTGV (SEQ ID NO:181)	30.000
6	10	ALLPAVPSL (SEQ ID NO:34)	9.000
7	439	QRNMTKLQL (SEQ ID NO:173)	7.500
8	390	RKFSRSDHL (SEQ ID NO:183)	6.000
9	396	DHLKTHTRT (SEQ ID NO:57)	6.000
10	239	NQMNLGATL (SEQ ID NO:151)	6.000
11	423	KKFARSDEL (SEQ ID NO:122)	6.000
12	126	RMFPNAPYL (SEQ ID NO:185)	6.000
13	225	NLYQMTSQL (SEQ ID NO:147)	6.000
14	180	DPMGQQGSL (SEQ ID NO:59)	6.000
15	144	IRNQGYSTV (SEQ ID NO:117)	5.000
16	136	SCLESQPAI (SEQ ID NO:198)	4.000
17	292	GVFRGIQDV (SEQ ID NO:103)	3.000
18	302	RVPGVAPTL (SEQ ID NO:195)	3.000
19	208	SCTGSQALL (SEQ ID NO:202)	3.000
20	207	DSCTGSQAL (SEQ ID NO:61)	3.000

Table XXIV
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 3902

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	239	NQMNLGATL (SEQ	24.000

		ID NO:151)	
2	390	RKFSRSDHL (SEQ ID NO:183)	20.000
3	423	KKFARSDEL (SEQ ID NO:122)	20.000
4	32	AQWAPVLDF (SEQ ID NO:37)	5.000
5	146	NQGYSTVTF (SEQ ID NO:150)	5.000
6	130	NAPYLPSCL (SEQ ID NO:144)	2.400
7	225	NLYQMTSQL (SEQ ID NO:147)	2.400
8	30	GAAQWAPVL (SEQ ID NO:86)	2.400
9	441	NMTKLQLAL (SEQ ID NO:149)	2.400
10	302	RVPGVAPTL (SEQ ID NO:195)	2.400
11	126	RMFPNAPYL (SEQ ID NO:185)	2.000
12	218	RTPYSSDNL (SEQ ID NO:194)	2.000
13	209	CTGSQALLL (SEQ ID NO:52)	2.000
14	332	KRYFKLSHL (SEQ ID NO:127)	2.000
15	180	DPMGQQGSL (SEQ ID NO:59)	2.000
16	437	MHQRNMTKL (SEQ ID NO:143)	2.000
17	207	DSCTGSQAL (SEQ ID NO:61)	2.000
18	208	SCTGSQALL (SEQ ID NO:202)	2.000
19	329	GCNKRYFKL (SEQ ID NO:90)	2.000
20	10	ALLPAVPSL (SEQ ID NO:34)	2.000

Table XXV
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 4403

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	315	SETSEKRPF (SEQ ID NO:209)	80.000
2	349	GEKPYQCDF (SEQ ID NO:91)	80.000
3	84	HEEQCLSAF (SEQ ID NO:107)	60.000
4	410	SEKPFSCRW (SEQ ID NO:207)	48.000
5	429	DELVRHHNM (SEQ ID NO:53)	24.000
6	278	TPILCGAQY (SEQ ID NO:227)	15.000
7	141	QPAIRNQGY (SEQ ID NO:170)	9.000
8	40	FAPPGASAY (SEQ ID NO:74)	9.000
9	213	QALLLRTPY (SEQ ID NO:160)	9.000
10	318	SEKRPFMCA (SEQ ID NO:208)	8.000
11	81	AEPHEEQCL (SEQ ID NO:30)	8.000
12	152	VTFDGTPSY (SEQ ID NO:244)	4.500
13	101	TGTAGACRY (SEQ ID NO:224)	4.500
14	120	ASSGQARMF (SEQ ID NO:40)	4.500
15	261	TEGQSNHST (SEQ ID NO:221)	4.000
16	85	EEQCLSAFT (SEQ ID NO:65)	4.000
17	233	LECMTWNQM (SEQ ID NO:131)	4.000
18	104	AGACRYGPF (SEQ ID NO:31)	4.000
19	3	SDVRDLNAL (SEQ	3.000

		ID NO:206)	
20	185	QGSLGEQQY (SEQ ID NO:166)	3.000

Table XXVI
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 5101

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	303	VPGVAPTLV (SEQ ID NO:242)	314.600
2	180	DPMGQQGSL (SEQ ID NO:59)	242.000
3	250	VAAGSSSSV (SEQ ID NO:236)	157.300
4	130	NAPYLPSCL (SEQ ID NO:144)	50.000
5	30	GAAQWAPVL (SEQ ID NO:86)	50.000
6	20	GGGGCALPV (SEQ ID NO:92)	44.000
7	64	PPPPPHSFI (SEQ ID NO:157)	40.000
8	29	SGAAQWAPV (SEQ ID NO:211)	40.000
9	18	LGGGGGCAL (SEQ ID NO:134)	31.460
10	295	RGIQDVRRV (SEQ ID NO:179)	22.000
11	119	QASSGQARM (SEQ ID NO:161)	18.150
12	418	WPSCQKKFA (SEQ ID NO:246)	12.100
13	82	EPHEEQCLS (SEQ ID NO:68)	12.100
14	110	GPFGPPPPS (SEQ ID NO:96)	11.000
15	272	ESDNHTTPI (SEQ ID NO:71)	8.000
16	306	VAPTLVRSA (SEQ ID NO:237)	7.150
17	280	ILCGAQYRI (SEQ ID	6.921

		NO:116)	
18	219	TPYSSDNLY (SEQ ID NO:231)	6.600
19	128	FPNAPYLPS (SEQ ID NO:79)	6.500
20	204	TPTDSCTGS (SEQ ID NO:230)	6.050

Table XXVII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 5102

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	295	RGIQDVRRV (SEQ ID NO:179)	290.400
2	303	VPGVAPTLV (SEQ ID NO:242)	200.000
3	180	DPMGQQGSL (SEQ ID NO:59)	133.100
4	250	VAAGSSSSV (SEQ ID NO:236)	110.000
5	30	GAAQWAPVL (SEQ ID NO:86)	55.000
6	130	NAPYLPSCL (SEQ ID NO:144)	50.000
7	20	GGGGCALPV (SEQ ID NO:92)	44.000
8	29	SGAAQWAPV (SEQ ID NO:211)	44.000
9	64	PPPPPHSFI (SEQ ID NO:157)	40.000
10	119	QASSGQARM (SEQ ID NO:161)	36.300
11	110	GPFGPPPPS (SEQ ID NO:96)	27.500
12	412	KPFSCRWPS (SEQ ID NO:123)	25.000
13	18	LGGGGGCAL (SEQ ID NO:134)	24.200
14	24	CALPVSGAA (SEQ ID NO:43)	16.500
15	219	TPYSSDNLY (SEQ ID	15.000

		NO:231)	
16	292	GVFRGIQDV (SEQ ID NO:103)	14.641
17	136	SCLESQPAI (SEQ ID NO:198)	14.520
18	418	WPSCQKKFA (SEQ ID NO:246)	12.100
19	269	TGYESDNHT (SEQ ID NO:225)	11.000
20	351	KPYQCDFKD (SEQ ID NO:124)	11.000

Table XXVIII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 5201

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	191	QQYSVPPPV (SEQ ID NO:171)	100.000
2	32	AQWAPVLDF (SEQ ID NO:37)	30.000
3	243	LGATLKGVA (SEQ ID NO:133)	16.500
4	303	VPGVAPTLV (SEQ ID NO:242)	13.500
5	86	EQCLSAFTV (SEQ ID NO:69)	12.000
6	295	RGIQDVRRV (SEQ ID NO:179)	10.000
7	98	GQFTGTAGA (SEQ ID NO:99)	8.250
8	292	GVFRGIQDV (SEQ ID NO:103)	8.250
9	29	SGAAQWAPV (SEQ ID NO:211)	6.000
10	146	NQGYSTVTF (SEQ ID NO:150)	5.500
11	20	GGGGCALPV (SEQ ID NO:92)	5.000
12	239	NQMNLGATL (SEQ ID NO:151)	4.000
13	64	PPPPPHSFI (SEQ ID	3.600

		NO:157)	
14	273	SDNHTTPIL (SEQ ID NO:204)	3.300
15	286	YRIHTHGVF (SEQ ID NO:252)	3.000
16	269	TGYESDNHT (SEQ ID NO:225)	3.000
17	406	TGKTSEKPF (SEQ ID NO:222)	2.750
18	327	YPGCNKRYF (SEQ ID NO:250)	2.750
19	7	DLNALLPAV (SEQ ID NO:58)	2.640
20	104	AGACRYGPF (SEQ ID NO:31)	2.500

Table XXIX
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 5801

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	230	TSQLECMTW (SEQ ID NO:234)	96.800
2	92	FTVHFSGQF (SEQ ID NO:85)	60.000
3	120	ASSGQARMF (SEQ ID NO:40)	40.000
4	168	AAQFPNHSF (SEQ ID NO:29)	20.000
5	408	KTSEKPFSC (SEQ ID NO:129)	12.000
6	394	RSDHLKTHT (SEQ ID NO:192)	9.900
7	276	HTTPILCGA (SEQ ID NO:115)	7.200
8	218	RTPYSSDNL (SEQ ID NO:194)	6.600
9	152	VTFDGTPSY (SEQ ID NO:244)	6.000
10	40	FAPPGASAY (SEQ ID NO:74)	6.000
11	213	QALLLRTPY (SEQ ID	4.500

		NO:160)	
12	347	HTGEKPYQC (SEQ ID NO:112)	4.400
13	252	AGSSSSVKW (SEQ ID NO:32)	4.400
14	211	GSQALLLRT (SEQ ID NO:102)	4.356
15	174	HSFKHEDPM (SEQ ID NO:110)	4.000
16	317	TSEKRPFMC (SEQ ID NO:233)	4.000
17	26	LPVSGAAQW (SEQ ID NO:138)	4.000
18	289	HTHGVFRGI (SEQ ID NO:113)	3.600
19	222	SSDNLYQMT (SEQ ID NO:217)	3.300
20	96	FSGQFTGTA (SEQ ID NO:82)	3.300

Table XXX
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA CW0301

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	10	ALLPAVPSL (SEQ ID NO:34)	100.000
2	332	KRYFKLSHL (SEQ ID NO:127)	48.000
3	126	RMFPNAPYL (SEQ ID NO:185)	36.000
4	3	SDVRDLNAL (SEQ ID NO:206)	30.000
5	239	NQMNLGATL (SEQ ID NO:151)	24.000
6	225	NLYQMTSQL (SEQ ID NO:147)	24.000
7	180	DPMGQQGSL (SEQ ID NO:59)	20.000
8	362	RRFSRSDQL (SEQ ID NO:187)	12.000
9	329	GCNKRYFKL (SEQ	10.000

		ID NO:90)	
10	286	YRIHTHGVF (SEQ ID NO:252)	10.000
11	301	RRVPGVAPT (SEQ ID NO:189)	10.000
12	24	CALPVSGAA (SEQ ID NO:43)	10.000
13	136	SCLESQPAI (SEQ ID NO:198)	7.500
14	437	MHQRNMTKL (SEQ ID NO:143)	7.200
15	390	RKFSRSDHL (SEQ ID NO:183)	6.000
16	423	KKFARSDEL (SEQ ID NO:122)	6.000
17	92	FTVHFSGQF (SEQ ID NO:85)	5.000
18	429	DELVRHHNM (SEQ ID NO:53)	5.000
19	130	NAPYLPSCL (SEQ ID NO:144)	4.800
20	30	GAAQWAPVL (SEQ ID NO:86)	4.000

Table XXXI
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA CW0401

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	356	DFKDCERRF (SEQ ID NO:55)	120.000
2	334	YFKLSHLQM (SEQ ID NO:248)	100.000
3	180	DPMGQQGSL (SEQ ID NO:59)	88.000
4	163	TPSHHAAQF (SEQ ID NO:228)	52.800
5	327	YPGCNKRYF (SEQ ID NO:250)	40.000
6	285	QYRIHTHGV (SEQ ID NO:175)	27.500
7	424	KFARSDELV (SEQ	25.000

		ID NO:119)	
8	326	AYPGCNKRY (SEQ ID NO:42)	25.000
9	192	QYSVPPPVY (SEQ ID NO:176)	25.000
10	417	RWPSCQKKF (SEQ ID NO:196)	22.000
11	278	TPILCGAQY (SEQ ID NO:227)	12.000
12	10	ALLPAVPSL (SEQ ID NO:34)	11.616
13	141	QPAIRNQGY (SEQ ID NO:170)	11.000
14	303	VPGVAPTLV (SEQ ID NO:242)	11.000
15	219	TPYSSDNLY (SEQ ID NO:231)	10.000
16	39	DFAPPGASA (SEQ ID NO:54)	7.920
17	99	QFTGTAGAC (SEQ ID NO:165)	6.000
18	4	DVRDLNALL (SEQ ID NO:62)	5.760
19	70	SFIKQEPSW (SEQ ID NO:210)	5.500
20	63	PPPPPPHSF (SEQ ID NO:158)	5.280

Table XXXII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA CW0602

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	332	KRYFKLSHL (SEQ ID NO:127)	9.680
2	239	NQMNLGATL (SEQ ID NO:151)	6.600
3	130	NAPYLPSCL (SEQ ID NO:144)	6.600
4	7	DLNALLPAV (SEQ ID NO:58)	6.000
5	441	NMTKLQLAL (SEQ	6.000

		ID NO:149)	
6	225	NLYQMTSQL (SEQ ID NO:147)	6.000
7	4	DVRDLNALL (SEQ ID NO:62)	6.000
8	3	SDVRDLNAL (SEQ ID NO:206)	4.400
9	10	ALLPAVPSL (SEQ ID NO:34)	4.000
10	213	QALLLRTPY (SEQ ID NO:160)	3.300
11	319	EKRPFMCAY (SEQ ID NO:67)	3.000
12	30	GAAQWAPVL (SEQ ID NO:86)	2.200
13	242	NLGATLKGV (SEQ ID NO:146)	2.200
14	292	GVFRGIQDV (SEQ ID NO:103)	2.200
15	207	DSCTGSQAL (SEQ ID NO:61)	2.200
16	362	RRFSRSDQL (SEQ ID NO:187)	2.200
17	439	QRNMTKLQL (SEQ ID NO:173)	2.200
18	295	RGIQDVRRV (SEQ ID NO:179)	2.200
19	423	KKFARSDEL (SEQ ID NO:122)	2.200
20	180	DPMGQQGSL (SEQ ID NO:59)	2.200

Table XXXIII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA CW0702

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	319	EKRPFMCAY (SEQ ID NO:67)	26.880
2	326	AYPGCNKRY (SEQ ID NO:42)	24.000
3	40	FAPPGASAY (SEQ	14.784

		ID NO:74)	
4	192	QYSVPPPVY (SEQ ID NO:176)	12.000
5	278	TPILCGAQY (SEQ ID NO:227)	12.000
6	219	TPYSSDNLY (SEQ ID NO:231)	12.000
7	213	QALLLRTPY (SEQ ID NO:160)	8.800
8	125	ARMFPNAPY (SEQ ID NO:38)	8.000
9	327	YPGCNKRYF (SEQ ID NO:250)	6.600
10	152	VTFDGTPSY (SEQ ID NO:244)	5.600
11	141	QPAIRNQGY (SEQ ID NO:170)	4.800
12	345	RKHTGEKPY (SEQ ID NO:184)	4.000
13	185	QGSLGEQQY (SEQ ID NO:166)	4.000
14	101	TGTAGACRY (SEQ ID NO:224)	4.000
15	375	RRHTGVKPF (SEQ ID NO:188)	4.000
16	263	GQSNHSTGY (SEQ ID NO:100)	4.000
17	163	TPSHHAAQF (SEQ ID NO:228)	3.000
18	33	QWAPVLDFA (SEQ ID NO:174)	2.688
19	130	NAPYLPSCL (SEQ ID NO:144)	2.640
20	84	HEEQCLSAF (SEQ ID NO:107)	2.400

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Table XXXIV

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Mouse MHC Class I Db

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	235	CMTWNQMNL (SEQ ID NO:49)	5255.712
2	126	RMFPNAPYL (SEQ ID NO:185)	1990.800
3	221	YSSDNLYQM (SEQ ID NO:253)	930.000
4	228	QMTSQLECM (SEQ ID NO:169)	33.701
5	239	NQMNLGATL (SEQ ID NO:151)	21.470
6	441	NMTKLQLAL (SEQ ID NO:149)	19.908
7	437	MHQRNMTKL (SEQ ID NO:143)	19.837
8	136	SCLESQPAI (SEQ ID NO:198)	11.177
9	174	HSFKHEDPM (SEQ ID NO:110)	10.800
10	302	RVPGVAPTL (SEQ ID NO:195)	10.088
11	130	NAPYLPSCL (SEQ ID NO:144)	8.400
12	10	ALLPAVPSL (SEQ ID NO:34)	5.988
13	208	SCTGSQALL (SEQ ID NO:202)	4.435
14	209	CTGSQALLL (SEQ ID NO:52)	3.548
15	238	WNQMNLGAT (SEQ ID NO:245)	3.300
16	218	RTPYSSDNL (SEQ ID NO:194)	3.185
17	24	CALPVSGAA (SEQ ID NO:43)	2.851
18	18	LGGGGGCAL (SEQ ID NO:134)	2.177

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19	142	PAIRNQGYS (SEQ ID NO:152)	2.160
20	30	GAAQWAPVL (SEQ ID NO:86)	1.680

Table XXXV
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Mouse MHC Class I Dd

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	112	FGPPPPSQA (SEQ ID NO:76)	48.000
2	122	SGQARMFPN (SEQ ID NO:212)	36.000
3	104	AGACRYGPF (SEQ ID NO:31)	30.000
4	218	RTPYSSDNL (SEQ ID NO:194)	28.800
5	130	NAPYLPSCL (SEQ ID NO:144)	20.000
6	302	RVPGVAPTL (SEQ ID NO:195)	20.000
7	18	LGGGGGCAL (SEQ ID NO:134)	20.000
8	81	AEPHEEQCL (SEQ ID NO:30)	10.000
9	29	SGAAQWAPV (SEQ ID NO:211)	7.200
10	423	KKFARSDEL (SEQ ID NO:122)	7.200
11	295	RGIQDVRRV (SEQ ID NO:179)	7.200
12	390	RKFSRSDHL (SEQ ID NO:183)	6.000
13	332	KRYFKLSHL (SEQ ID NO:127)	6.000
14	362	RRFSRSDQL (SEQ ID NO:187)	6.000
15	417	RWPSCQKKF (SEQ ID NO:196)	6.000
16	160	YGHTPSHHA (SEQ ID NO:249)	6.000

17	20	GGGGCALPV (SEQ ID NO:92)	6.000
18	329	GCNKRYFKL (SEQ ID NO:90)	5.000
19	372	RHQRRHTGV (SEQ ID NO:181)	4.500
20	52	GGPAPPPAP (SEQ ID NO:93)	4.000

Table XXXVI
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Mouse MHC Class I Kb

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	329	GCNKRYFKL (SEQ ID NO:90)	24.000
2	225	NLYQMTSQL (SEQ ID NO:147)	10.000
3	420	SCQKKFARS (SEQ ID NO:200)	3.960
4	218	RTPYSSDNL (SEQ ID NO:194)	3.630
5	437	MHQRNMTKL (SEQ ID NO:143)	3.600
6	387	TCQRKFSRS (SEQ ID NO:219)	3.600
7	302	RVPGVAPTL (SEQ ID NO:195)	3.300
8	130	NAPYLPSCL (SEQ ID NO:144)	3.000
9	289	HTHGVFRGI (SEQ ID NO:113)	3.000
10	43	PGASAYGSL (SEQ ID NO:153)	2.400
11	155	DGTPSYGHT (SEQ ID NO:56)	2.400
12	273	SDNHTTPIL (SEQ ID NO:204)	2.200
13	126	RMFPNAPYL (SEQ ID NO:185)	2.200
14	128	FPNAPYLPS (SEQ ID NO:79)	2.000

15	3	SDVRDLNAL (SEQ ID NO:206)	1.584
16	207	DSCTGSQAL (SEQ ID NO:61)	1.584
17	332	KRYFKLSHL (SEQ ID NO:127)	1.500
18	18	LGGGGGCAL (SEQ ID NO:134)	1.320
19	233	LECMTWNQM (SEQ ID NO:131)	1.320
20	441	NMTKLQLAL (SEQ ID NO:149)	1.200

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Table XXXVII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Mouse MHC Class I Kd

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	285	QYRIHTHGV (SEQ ID NO:175)	600.000
2	424	KFARSDELV (SEQ ID NO:119)	288.000
3	334	YFKLSHLQM (SEQ ID NO:248)	120.000
4	136	SCLESQPTI (SEQ ID NO:199)	115.200
5	239	NQMNLGATL (SEQ ID NO:151)	115.200
6	10	ALLPAVSSL (SEQ ID NO:35)	115.200
7	47	AYGSLGGPA (SEQ ID NO:41)	86.400
8	180	DPMGQQGSL (SEQ ID NO:59)	80.000
9	270	GYESDNHTA (SEQ ID NO:105)	72.000
10	326	AYPGCNKRY (SEQ ID NO:42)	60.000
11	192	QYSVPPPVY (SEQ ID NO:176)	60.000
12	272	ESDNHTAPI (SEQ ID NO:70)	57.600
13	289	HTHGVFRGI (SEQ ID NO:113)	57.600
14	126	DVRDLNALL (SEQ ID NO:62)	57.600
15	4	CTGSQALLL (SEQ ID NO:52)	57.600
16	208	SCTGSQALL (SEQ ID NO:202)	48.000
17	441	NMTKLQLAL (SEQ ID NO:149)	48.000
18	207	DSCTGSQAL (SEQ ID NO:61)	48.000
19	130	NAPYLPSCL (SEQ ID	48.000

		NO:144)	
20	235	CMTWNQMNL (SEQ ID NO:49)	48.000

Table XXXVIII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Mouse MHC Class I Kk

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	81	AEPHEEQCL (SEQ ID NO:30)	40.000
2	85	EEQCLSAFT (SEQ ID NO:65)	40.000
3	429	DELVRHHNM (SEQ ID NO:53)	20.000
4	315	SETSEKRPF (SEQ ID NO:209)	20.000
5	261	TEGQSNHST (SEQ ID NO:221)	20.000
6	410	SEKPFSCRW (SEQ ID NO:207)	10.000
7	272	ESDNHTTPI (SEQ ID NO:71)	10.000
8	318	SEKRPFMCA (SEQ ID NO:208)	10.000
9	138	LESQPAIRN (SEQ ID NO:132)	10.000
10	233	LECMTWNQM (SEQ ID NO:131)	10.000
11	298	QDVRRVPGV (SEQ ID NO:164)	10.000
12	84	HEEQCLSAF (SEQ ID NO:107)	10.000
13	349	GEKPYQCDF (SEQ ID NO:91)	10.000
14	289	HTHGVFRGI (SEQ ID NO:113)	10.000
15	179	EDPMGQQGS (SEQ ID NO:64)	8.000
16	136	SCLESQPAI (SEQ ID NO:198)	5.000
17	280	ILCGAQYRI (SEQ ID	5.000

		NO:116)	
18	273	SDNHTTPIL (SEQ ID NO:204)	4.000
19	428	SDELVRHHN (SEQ ID NO:203)	4.000
20	3	SDVRDLNAL (SEQ ID NO:206)	4.000

Table XXXIX
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Mouse MHC Class I Ld

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	163	TPSHHAAQF (SEQ ID NO:228)	360.000
2	327	YPGCNKRYF (SEQ ID NO:250)	300.000
3	180	DPMGQQGSL (SEQ ID NO:59)	150.000
4	26	LPVSGAAQW (SEQ ID NO:138)	93.600
5	278	TPILCGAQY (SEQ ID NO:227)	72.000
6	141	QPAIRNQGY (SEQ ID NO:170)	60.000
7	219	TPYSSDNLY (SEQ ID NO:231)	60.000
8	303	VPGVAPTLV (SEQ ID NO:242)	60.000
9	120	ASSGQARMF (SEQ ID NO:40)	50.000
10	63	PPPPPPHSF (SEQ ID NO:158)	45.000
11	113	GPPPPSQAS (SEQ ID NO:97)	45.000
12	157	TPSYGHTPS (SEQ ID NO:229)	39.000
13	207	DSCTGSQAL (SEQ ID NO:61)	32.500
14	110	GPFGPPPPS (SEQ ID NO:96)	30.000
15	82	EPHEEQCLS (SEQ ID	30.000

		NO:68)	
16	412	KPFSCRWPS (SEQ ID NO:123)	30.000
17	418	WPSCQKKFA (SEQ ID NO:246)	30.000
18	221	YSSDNLYQM (SEQ ID NO:253)	30.000
19	204	TPTDSCTGS (SEQ ID NO:230)	30.000
20	128	FPNAPYLPS (SEQ ID NO:79)	30.000

Table XL
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Cattle HLA A20

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	350	EKPYQCDFK (SEQ ID NO:66)	1000.00
2	319	EKRPFMCAY (SEQ ID NO:67)	500.000
3	423	KKFARSDEL (SEQ ID NO:122)	500.000
4	345	RKHTGEKPY (SEQ ID NO:184)	500.000
5	390	RKFSRSDHL (SEQ ID NO:183)	500.000
6	137	CLESQPAIR (SEQ ID NO:47)	120.000
7	380	VKPFQCKTC (SEQ ID NO:239)	100.000
8	407	GKTSEKPFS (SEQ ID NO:95)	100.000
9	335	FKLSHLQMH (SEQ ID NO:78)	100.000
10	247	LKGVAAGSS (SEQ ID NO:135)	100.000
11	370	LKRHQRRHT (SEQ ID NO:136)	100.000
12	258	VKWTEGQSN (SEQ ID NO:240)	100.000
13	398	LKTHTRTHT (SEQ	100.000

		ID NO:137)	
14	331	NKRYFKLSH (SEQ ID NO:145)	100.000
15	357	FKDCERRFS (SEQ ID NO:77)	100.000
16	385	CKTCQRKFS (SEQ ID NO:46)	100.000
17	294	FRGIQDVRR (SEQ ID NO:81)	80.000
18	368	DQLKRHQRR (SEQ ID NO:60)	80.000
19	432	VRHHNMHQR (SEQ ID NO:243)	80.000
20	118	SQASSGQAR (SEQ ID NO:216)	80.000

Table XLI
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Mouse WT1 Peptides to Mouse MHC Class I A_0201

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	126	RMFPNAPYL (SEQ ID NO:293)	313.968
2	187	SLGEQQYSV (SEQ ID NO:299)	285.163
3	10	ALLPAVSSL (SEQ ID NO:255)	181.794
4	225	NLYQMTSQL (SEQ ID NO:284)	68.360
5	292	GVFRGIQDV (SEQ ID NO:270)	51.790
6	93	TLHFSGQFT (SEQ ID NO:302)	40.986
7	191	QQYSVPPPV (SEQ ID NO:290)	22.566
8	280	ILCGAQYRI (SEQ ID NO:274)	17.736
9	441	NMTKLHVAL (SEQ ID NO:285)	15.428
10	235	CMTWNQMNL (SEQ ID NO:258)	15.428
11	7	DLNALLPAV (SEQ	11.998

		ID NO:261)	
12	242	NLGATLKGM (SEQ ID NO:283)	11.426
13	227	YQMTSQLEC (SEQ ID NO:307)	8.573
14	239	NQMNLGATL (SEQ ID NO:286)	8.014
15	309	TLVRSASET (SEQ ID NO:303)	7.452
16	408	KTSEKPFSC (SEQ ID NO:277)	5.743
17	340	LQMHSRKHT (SEQ ID NO:280)	4.752
18	228	QMTSQLECM (SEQ ID NO:289)	4.044
19	37	VLDFAPPGA (SEQ ID NO:304)	3.378
20	302	RVSGVAPTL (SEQ ID NO:295)	1.869

Table XLII
Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Mouse WT1 Peptides to Mouse MHC Class I Db

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	221	YSSDNLYQM (SEQ ID NO:308)	312.000
2	126	RMFPNAPYL (SEQ ID NO:293)	260.000
3	235	CMTWNQMNL (SEQ ID NO:258)	260.000
4	437	MHQRNMTKL (SEQ ID NO:281)	200.000
5	238	WNQMNLGAT (SEQ ID NO:305)	12.000
6	130	NAPYLPSCL (SEQ ID NO:282)	8.580
7	3	SDVRDLNAL (SEQ ID NO:298)	7.920
8	136	SCLESQPTI (SEQ ID NO:296)	7.920

9	81	AEPHEEQCL (SEQ ID NO:254)	6.600
10	10	ALLPAVSSL (SEQ ID NO:255)	6.600
11	218	RTPYSSDNL (SEQ ID NO:294)	6.000
12	441	NMTKLHVAL (SEQ ID NO:285)	3.432
13	228	QMTSQLECM (SEQ ID NO:289)	3.120
14	174	HSFKHEDPM (SEQ ID NO:272)	3.120
15	242	NLGATLKGM (SEQ ID NO:283)	2.640
16	261	TEGQSNHGI (SEQ ID NO:301)	2.640
17	225	NLYQMTSQL (SEQ ID NO:284)	2.640
18	207	DSCTGSQAL (SEQ ID NO:263)	2.600
19	119	QASSGQARM (SEQ ID NO:288)	2.600
20	18	LGGGGGCGL (SEQ ID NO:279)	2.600

Table XLIII

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Mouse WT1 Peptides to Mouse MHC Class I Kb

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	329	GCNKRYFKL (SEQ ID NO:268)	24.000
2	225	NLYQMTSQL (SEQ ID NO:284)	10.000
3	420	SCQKKFARS (SEQ ID NO:297)	3.960
4	218	RTPYSSDNL (SEQ ID NO:294)	3.630
5	437	MHQRNMTKL (SEQ ID NO:281)	3.600
6	387	TCQRKFSRS (SEQ ID	3.600

		NO:300)	
7	289	HTHGVFRGI (SEQ ID NO:273)	3.000
8	130	NAPYLPSCL (SEQ ID NO:282)	3.000
9	43	PGASAYGSL (SEQ ID NO:287)	2.400
10	155	DGAPSYGHT (SEQ ID NO:260)	2.400
11	126	RMFPNAPYL (SEQ ID NO:293)	2.200
12	128	FPNAPYLPS (SEQ ID NO:267)	2.000
13	207	DSCTGSQAL (SEQ ID NO:263)	1.584
14	3	SDVRDLNAL (SEQ ID NO:298)	1.584
15	332	KRYFKLSHL (SEQ ID NO:276)	1.500
16	233	LECMTWNQM (SEQ ID NO:278)	1.320
17	18	LGGGGGCGL (SEQ ID NO:279)	1.320
18	242	NLGATLKGM (SEQ ID NO:283)	1.200
19	123	GQARMFPN (SEQ ID NO:269)A	1.200
20	441	NMTKLHVAL (SEQ ID NO:285)	1.200

Table XLIV

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Mouse WT1 Peptides to Mouse MHC Class I Kd

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	285	QYRIHTHGV (SEQ ID NO:291)	600.000
2	424	KFARSDELV (SEQ ID NO:275)	288.000
3	334	YFKLSHLQM (SEQ ID NO:306)	120.000

4	136	SCLESQPTI (SEQ ID NO:296)	115.200
5	239	NQMNLGATL (SEQ ID NO:286)	115.200
6	10	ALLPAVSSL (SEQ ID NO:255)	115.200
7	47	AYGSLGGPA (SEQ ID NO:256)	86.400
8	180	DPMGQQGSL (SEQ ID NO:262)	80.000
9	270	GYESDNHTA (SEQ ID NO:271)	72.000
10	192	QYSVPPPVY (SEQ ID NO:292)	60.000
11	326	AYPGCNKRY (SEQ ID NO:257)	60.000
12	289	HTHGVFRGI (SEQ ID NO:273)	57.600
13	4	DVRDLNALL (SEQ ID NO:264)	57.600
14	126	RMFPNAPYL (SEQ ID NO:293)	57.600
15	209	CTGSQALLL (SEQ ID NO:259)	48.000
16	86	EQCLSAFTL (SEQ ID NO:265)	48.000
17	302	RVSGVAPTL (SEQ ID NO:295)	48.000
18	218	RTPYSSDNL (SEQ ID NO:294)	48.000
19	272	ESDNHTAPI (SEQ ID NO:266)	48.000
20	225	NLYQMTSQL (SEQ ID NO:284)	48.000

Table XLV

Results of TSites Peptide Binding Prediction Analysis for

Human WT1 Peptides Capable of Eliciting a Helper T cell Response

Peptide	Sequence
p6-23	RDLNALLPAVPSLGGGG (SEQ ID NO:1)
p30-35	GAAQWA (SEQ ID NO:309)
p45-56	ASAYGSLGGPAP (SEQ ID NO:310)

p91-105	AFTVHFSGQFTGTAG (SEQ ID NO:311)
p117-139	PSQASSGQARMFPNAPYLPSCLE (SEQ ID NO:2)
p167-171	HAAQF (SEQ ID NO:312)
p202-233	CHTPTDSCTGSQALLLRTPYSSDNLYQMTSQL (SEQ ID NO:313)
p244-262	GATLKGVAAGSSSSVKWTE (SEQ ID NO:4)
p287-318	RIHTHGVFRGIQDVRRVPGVAPTLVRSASETS (SEQ ID NO:314)
p333-336	RYFK (SEQ ID NO:315)
p361-374	ERRFSRSDQLKRHQ (SEQ ID NO:316)
p389-410	QRKFSRSDHLKTHTRTHTGKTS (SEQ ID NO:317)
p421-441	CQKKFARSDELVRHHNMHQRN (SEQ ID NO:318)

Certain CTL peptides (shown in Table XLVI) were selected for further study. For each peptide in Table XLVI, scores obtained using BIMAS HLA peptide binding prediction analysis are provided.

Table XLVI
WT1 Peptide Sequences and HLA Peptide Binding Predictions

Peptide	Sequence	Comments
p329-337	GCNKRYFKL (SEQ ID NOs: 90 and 268)	Score 24,000
p225-233	NLYQMTSQL (SEQ ID NOs: 147 and 284)	binds also to class II and HLA A2, Kd, score 10,000
p235-243	CMTWNQMNL (SEQ ID NOs: 49 and 258)	binds also to HLA A2, score 5,255,712
p126-134	RMFPNAPYL (SEQ ID NOs: 185 and 293)	binds also to Kd, class II and HLA A2, score 1,990,800
p221-229	YSSDNLYQM (SEQ ID NOs: 253 and 308)	binds also to Ld, score 312,000
p228-236	QMTSQLECM (SEQ ID NOs: 169 and 289)	score 3,120
p239-247	NQMNLGATL (SEQ ID NOs: 151 and 286)	binds also to HLA A 0201, Kd, score 8,015
mouse p136-144	SCLESQPTI (SEQ ID NO:296)	binds also to Kd, 1mismatch to human

human p136-144	SCLESQPAI (SEQ ID NO:198)	score 7,920
mouse p10-18	ALLPAVSSL (SEQ ID NO:255)	binds also to Kd, HLA A2, 1 mismatch to human
human p10-18	ALLPAVPSL (SEQ ID NO:34)	score 6,600

Peptide binding to C57Bl/6 murine MHC was confirmed using the RMA-S binding assay. Mice were immunized with the peptides capable of binding to murine class I MHC. Following immunization, spleen cells were stimulated *in vitro* and tested for the ability to lyse targets incubated with WT1 peptides. CTL were evaluated with a standard chromium release assay (Chen et al., *Cancer Res. 54*:1065-1070, 1994). The results, presented in Table XLVII, show that some WT1 peptides can bind to class I MHC molecules, which is essential for generating CTL. Moreover, several of the peptides were able to elicit peptide specific CTL (Figures 6A and 6B), as determined using chromium release assays. Following immunization to CTL peptides p10-18 human, p136-144 human, p136-144 mouse and p235-243, peptide specific CTL lines were generated and clones were established. These results indicate that peptide specific CTL can kill malignant cells expressing WT1.

Table XLVII
Binding of WT1 CTL Peptides to mouse B6 class I antigens

Peptide	Binding Affinity to Mouse MHC Class I
Positive control	91%
negative control	0.5.-1.3%
p235-243	33.6%
p136-144 mouse	27.9%
p136-144 human	52%
p10-18: human	2.2%
p225-233	5.8%
p329-337	1.2%
p126-134	0.9%
p221-229	0.8%
p228-236	1.2%
p239-247	1%

Example 5

Use of a WT1 Polypeptide to Elicit WT1 Specific CTL in Mice

This Example illustrates the ability of a representative WT1 polypeptide to elicit CTL immunity capable of killing WT1 positive tumor cell lines.

P117-139, a peptide with motifs appropriate for binding to class I and class II MHC, was identified as described above using TSITES and BIMAS HLA peptide binding prediction analyses. Mice were immunized as described in Example 3. Following immunization, spleen cells were stimulated *in vitro* and tested for the ability to lyse targets incubated with WT1 peptides, as well as WT1 positive and negative tumor cells. CTL were evaluated with a standard chromium release assay. The results, presented in Figures 7A-7D, show that P117 can elicit WT1 specific CTL capable of killing WT1 positive tumor cells, whereas no killing of WT1 negative cells was observed. These results demonstrate that peptide specific CTL in fact kill malignant cells expressing WT1 and that vaccine and T cell therapy are effective against malignancies that express WT1.

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Example 6

Identification of WT1 Specific mRNA in Mouse Tumor Cell Lines

This Example illustrates the use of RT-PCR to detect WT1 specific mRNA in cells and cell lines.

Mononuclear cells were isolated by density gradient centrifugation, and were immediately frozen and stored at -80°C until analyzed by RT-PCR for the presence of WT1 specific mRNA. Total RNA was extracted from 10^7 cells according to standard procedures. RNA pellets were resuspended in 25 μL diethylpyrocarbonate treated water and used directly for reverse transcription. Amplification was performed in a thermocycler during one or, when necessary, two sequential rounds of PCR. AmpliTaq DNA Polymerase (Perkin Elmer Cetus, Norwalk, CT), 2.5 mM $MgCl_2$ and

20 pmol of each primer in a total reaction volume of 50µl were used. Twenty µL aliquots of the PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide. The gels were photographed with Polaroid film (Polaroid 667, Polaroid Ltd., Hertfordshire, England). Precautions against cross contamination were taken following the recommendations of Kwok and Higuchi, *Nature 339*:237-238, 1989. Negative controls included the cDNA- and PCR-reagent mixes with water instead of cDNA in each experiment. To avoid false negatives, the presence of intact RNA and adequate cDNA generation was evaluated for each sample by a control PCR using β-actin primers. Samples that did not amplify with these primers were excluded from analysis.

Primers for amplification of WT1 in mouse cell lines were: P115: 1458-1478: 5' CCC AGG CTG CAA TAA GAG ATA 3' (forward primer; SEQ ID NO:21); and P116: 1767-1787: 5' ATG TTG TGA TGG CGG ACC AAT 3' (reverse primer; SEQ ID NO:22) (*see* Inoue et al, *Blood 88*:2267-2278, 1996; Fraizer et al., *Blood 86*:4704-4706, 1995).

Beta Actin primers used in the control reactions were: 5' GTG GGG CGC CCC AGG CAC CA 3' (sense primer; SEQ ID NO:23); and 5' GTC CTT AAT GTC ACG CAC GAT TTC 3' (antisense primer; SEQ ID NO:24)

Primers for use in amplifying human WT1 include: P117: 954-974: 5' GGC ATC TGA GAC CAG TGA GAA 3' (SEQ ID NO:25); and P118: 1434-1414: 5' GAG AGT CAG ACT TGA AAG CAGT 3' (SEQ ID NO:5). For nested RT-PCR, primers may be: P119: 1023-1043: 5' GCT GTC CCA CTT ACA GAT GCA 3' (SEQ ID NO:26); and P120: 1345-1365: 5' TCA AAG CGC CAG CTG GAG TTT 3' (SEQ ID NO:27).

Table XLVIII shows the results of WT1 PCR analysis of mouse tumor cell lines. Within Table IV, (+++) indicates a strong WT1 PCR amplification product in the first step RT PCR, (++) indicates a WT1 amplification product that is detectable by first step WT1 RT PCR, (+) indicates a product that is detectable only in the second step of WT1 RT PCR, and (-) indicates WT1 PCR negative.

Table XLVIII
Detection of WT1 mRNA in Mouse Tumor Cell Lines

Cell Line	WT1 mRNA
K562 (human leukemia; ATCC): Positive control; (Lozzio and Lozzio, *Blood 45*:321-334, 1975)	+++
TRAMPC (SV40 transformed prostate, B6); Foster et al., *Cancer Res. 57*:3325-3330, 1997	+++
BLK SV40 HD2 (SV40-transf. fibroblast, B6; ATCC); *Nature 276*:510-511, 1978	++
CTLL (T-cell, B6; ATCC); Gillis, *Nature 268*:154-156, 1977)	+
FM (FBL-3 subline, leukemia, B6); Glynn and Fefer, *Cancer Res. 28*:434-439, 1968	+
BALB 3T3 (ATCC); Aaroston and Todaro, *J. Cell. Physiol. 72*:141-148, 1968	+
S49.1 (Lymphoma, T-cell like, B/C; ATCC); Horibata and Harris, *Exp. Cell. Res. 60*:61, 1970	+
BNL CL.2 (embryonic liver, B/C; ATCC); *Nature 276*:510-511, 1978	+
MethA (sarcoma, B/C); Old et al., *Ann. NY Acad. Sci. 101*:80-106, 1962	-
P3.6.2.8.1 (myeloma, B/C; ATCC); *Proc. Natl. Acad. Sci. USA 66*:344, 1970	-
P2N (leukemia, DBA/2; ATCC); Melling et al., *J. Immunol. 117*:1267-1274, 1976	-
BCL1 (lymphoma, B/C; ATCC); Slavin and Strober, *Nature 272*:624-626, 1977	-
LSTRA (lymphoma, B/C); Glynn et al., *Cancer Res. 28*:434-439, 1968	-
E10 (lymphoma, B6); Glynn et al., *Cancer Res. 28*:434-439, 1968	-

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.